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L5            25 S L4 NOT L1
L6            24 S L4 NOT L3
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L8         71131 S (VECTOR?/CLM)
L9          3255 S L8 AND (RETROVIR?/CLM OR LENTIVIR?/CLM OR FIV/CLM OR EIAV/CLM
L10          134 S L9 AND (CMV PROMOTER/CLM OR H4 PROMOTER/CLM OR PGK PROMOTER/C
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L13            6 S L12 AND (SECOND REPORTER)
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L46           20 S L45 AND P21
L47           13 S L46 AND PY<2004
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- ASSIGNMENT DEFICIENT

09/08/03

IN THE CLAIMS: GRAPHIC (1-24) W/OTRANSF

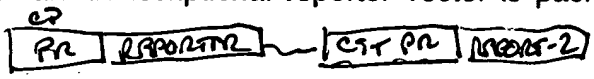
Please amend the claims as follows: SEE PARENT 10/658,552

102(b) TROVO NAKA JOHN

(1) (Original) A viral transcriptional reporter vector comprising:

a vector backbone derived from a virus of the family Retroviridae;

a conditional promoter and a first reporter cassette under control of the conditional promoter, wherein said transcriptional reporter vector is packaged in viral particles.



102(b) TROVO NAKA JOHN

(2) (Original) The viral transcriptional reporter vector of claim 1, wherein the virus is a lentivirus.

(3) (Original) The viral transcriptional reporter vector of claim 1, further comprising a constitutive promoter and a second reporter cassette under the control of the constitutive promoter, wherein said first and second reporter cassettes generate distinguishable effects in biological assays.

103: TROVO 3-9 11

(4) (Original) The viral transcriptional reporter vector of claim 3, wherein the reporter cassettes generate fluorescent signals, colorimetric signals, or combinations thereof.

(5) (Original) The viral transcriptional reporter vector of claim 4, wherein the second reporter cassette is an RFP cassette.

(6) (Original) The viral transcriptional reporter vector of claim 5, wherein the first reporter cassette comprises a coding region selected from the group consisting of beta-galactosidase coding regions or GFP coding regions.

(7) (Currently Amended) The viral transcriptional reporter vector of claim [[1]] 3, wherein the first and second reporter cassettes comprise coding regions for two different fluorescent proteins.

INVENT M. VERMA
DEPEND TROVO
ALAN J KLEIDERMAN
DOUGLAS DOLLY

LENTIVIRAL VECTOR
GFP/RFP REPORTER VECTOR
pLSLP VECTOR
H4 PROMOTER

8. (Original) The viral transcriptional reporter vector of claim 7, wherein the two different fluorescent proteins are GFP and BFP.

9. (Original) The viral transcriptional reporter vector of claim 1, wherein the retrovirus-derived backbone is FIV-based.

10. (Original) The viral transcriptional reporter vector of claim 1, wherein the retrovirus-derived backbone is derived from HIV, visan-maedi, caprine arthritis-encephalitis virus, EIAV, BIV, or SIV.

11. (Currently Amended) The viral transcriptional reporter vector of claim [[1]] 3, wherein the ~~constitutive promoter~~ is selected from the group consisting of a histone H4 promoter, a minimal immediately early promoter of cytomegalovirus, a pgk promoter, an EF-1 alfa promoter, and a ubiquitin promoter.

12. (Original) The viral transcriptional reporter vector of claim 1, further comprising sequences from the 5' and 3' LTRs of a retrovirus.

13. (Original) The viral transcriptional reporter vector of claim 1, further comprising a central polypurine tract of a retroviral polymerase gene.

14. (Original) A viral transcriptional reporter vector comprising:

a lentivirus-derived vector backbone; and

a conditional promoter comprising one or more pathway-specific response elements and a first reporter cassette under the control of the conditional promoter.

15. (Original) The viral transcriptional reporter vector of claim 14, wherein the one or more pathway-specific response elements comprise a p53 binding sequence.

16. (Original) The viral transcriptional reporter vector of claim 15, wherein the one or more pathway-specific response elements comprise p53 binding sequences from p21.

17. (Original) The viral transcriptional reporter vector of claim 14, wherein the conditional promoter further comprises a minimal immediate early promoter of cytomegalovirus.

18. (Original) The viral transcriptional reporter vector of claim 14, further comprising a constitutive promoter and a second reporter cassette under the control of the constitutive promoter.

19. (Original) The viral transcriptional reporter vector of claim 18, wherein the reporter cassettes produce colorimetric signals, fluorescent signals, luminescent signals or combinations thereof in cell-based assays.

20. (Original) The viral transcriptional reporter vector of claim 18, wherein the first and second reporter cassettes comprise coding regions for two different fluorescent proteins.

21. (Original) The viral transcriptional reporter vector of claim 14, wherein the lentivirus-derived backbone is FIV-based.

22. (Currently Amended) The viral transcriptional reporter vector of claim 14, wherein the lentivirus backbone is derived from at least one of HIV, visan-maedi, caprine arthritis-encephalitis virus, EIAV, BIV, and SIV.

23. (Original) The viral transcriptional reporter vector of claim 14, further comprising sequences from the 5' and 3' LTRs of a lentivirus.

24. (Original) The viral transcriptional reporter vector of claim 14, further comprising a central polypurine tract of a lentiviral polymerase gene.

25. (Withdrawn) A reporter cell generated by transduction of a cell with the packaged viral transcriptional reporter vector of claim 1.

26. (Withdrawn) The reporter cell of claim 25, wherein the viral reporter vector is stably integrated into the reporter cell's genome.

27. (Withdrawn) The reporter cell of claim 25, wherein the viral transcriptional reporter vector further comprises a constitutive promoter and a second reporter cassette under the control of the constitutive promoter.

28-33. (Canceled)

3/12

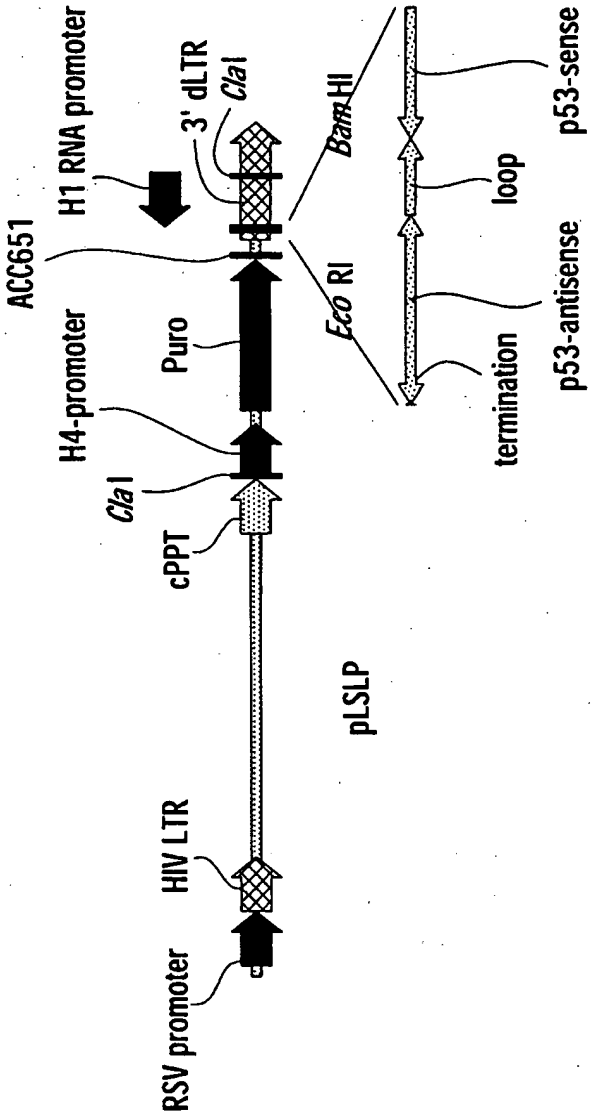


FIG. 3

MARCH

MARCH 19

NV
CMV
GFP

6096538 RNVP
6312683

- 6068982 p53 PROMOTER IN REPORTER

* US2002/0094517 p53 FROM p21

5874304 GFP

6410013 — RRP-1 (β-GAL, GFP)
RRP-2

PNAS- 94(19): 10319 1997

TWO PROMOTERS
DUAL REPORTER
MULTIPLE REPORTER
DOUBLE PROMOTER

US 2003/0101472 - RNVP
GFP
CONST./COND. PR

US 2003/0138954 - RNVP
GFP
CONST/COND PR
CPPT

US2002/0037281

FIV US 2002/0048805

LacZ-H4-puro, has been developed. This reporter vector was originally designed to screen for chemical compounds that might activate a p53-dependent pathway using induction of beta-galactosidase as a reporter. The lentiviral backbone of the pL-p53RE-LacZ-H4-puro vector was derived from a self-inactivating lentiviral vector pLV-GFP (Pfeifer, et al., PNAS, 99: 2140 (2002)). The pL-p53RE-LacZ-H4-puro vector was engineered to provide high viral titer and to inactivate the promoter in the 5' LTR following integration into the host genome. Self-inactivating vectors provide more consistent and improved expression due to reduced promoter interference, and self-inactivating lentiviral vectors are safer to work with because they are less likely to form replication-competent retrovirus.

[0092] In order to construct the pL-p53RE-LacZ-H4-puro vector, a synthetic multiple cloning site (MCS) was inserted into a basic lentiviral backbone and the following elements were cloned into the MCS: a 20 bp high-affinity consensus p53 binding sequence, a 43 bp "fragment A" from the ribosome gene cluster containing several p53-binding sites, six copies of a p53-binding site from a human gene for the CDK inhibitor p21WAF1/Cip1 (Deiry, et all, Cell, 75:815 (1993)), the minimal immediate early promoter of cytomegalovirus (mCMV), a bacterial LacZ gene encoding beta-galactosidase, and a puromycin-resistance gene under control of constitutive promoter of histone H4. The XbaI-SpeI sites were used for directional cloning of various novel pathway-specific response elements. In addition, the XhoI and BamHI sites were used, for example on one embodiment, for the cloning of a destabilized GFP reporter gene. A dsRed reporter gene could replace the puro gene using NheI-Acc65I sites in the same or yet another embodiment.

[0093] In order to test the performance of this reporter vector in gene silencing experiments, the pL-p53RE-LacZ-H4-puro construct was packaged in pseudoviral particles by cotransfection with a pCMV-delta8.2 plasmid and the pVSV-G plasmid, which express the VSV-G envelope protein in the 293T packaging cell line. After 48 hours, the virus-containing supernatants were collected and used for infection of HeLa cells. A stable p53 transcriptional reporter cell line was generated after selection of puromicine-resistant HeLa cells.

without a stem-loop structure. As a result of *in vivo* transcription, the resulting siRNA has a double-stranded structure without additional non-mRNA sequences, with the exception of the initiation nucleotide (G) and a (U)2-3 overhang at the 3' ends.

[0082] The double-stranded siRNA constructs were found to provide similar silencing efficiency similar to hairpin-type siRNA constructs designed for the same 27-bp p53 sequence and tested in the luciferase assay as described above. Replacement of the wild-type promoter sequences with a termination signal just upstream of the transcriptional start site was found not to change activity of the promoters. The dual promoter construct has been found to be more stable than the stem-loop construct during the propogation in *E. coli* and mammalian cells.

[0083] In another embodiment, the siRNA library vector was based on a biologically safe FIV viral vector developed at UCSD (US Patent 6,555,107 B2). One reason for the selection of a non-primate FIV-based vector system and development of FIV-based products is due to safety. The pFIV vectors have a structure very similar to pL-based vectors just described. Specific blocks of the FIV genome were used to replace the corresponding HIV elements in the pLSLP vectors.

[0084] The resulting pFIV siRNA cloning vectors have several important features. First, the FIV siRNA vector contains a hybrid RSV/FIV promoter in the 5'-LTR with lentiviral R and U5 sequences for efficient control of vector packaging and integration. Also, the FIV siRNA vector contains a deletion in the enhancer of the U3 region of 3'-LTR to provide a self-inactivating property to the reporter vector. In addition, the pFIV siRNA cloning vector contains the region of the FIV genome required for efficient packaging: 1) a central purine pyrimidine tract (cPPT) derived from the FIV genome that is important for improved nuclear translocation in non-dividing cells (which increases the efficiency of gene transfer); 2) internal expression cassettes controlling fluorescent reporter protein or drug resistance gene flanked by unique restriction sites; and 3) unique restriction sites providing a modular structure (facilitating interchangeable replacement of reporter genes, drug resistance genes, transcription factor response elements and minimal promoters).

ABI provide about 10,000 500-5000-mer cDNAs or 4,000 – 35,000 45-80-mer oligonucleotides printed by ink-jet printer or by pins onto glass slides, which are also analyzed by fluorescence, radioactivity or chemiluminescense. Essentially, microarrays can be made by photolithography, spotting oligonucleotides synthesized by standard phosphoramidite chemistry, photochemistry, electrochemistry, or the like. Analysis techniques include fluorescence, mass spectrometry, chemiluminescense or radioisotopic methods.

Example 1. Development of lentiviral vectors for cloning effector libraries.

[0076] In order to develop an efficient siRNA library transduction vector; a pL-reporter lentiviral backbone was used in which the puromycin-resistance gene is controlled by H4 promoter (see Fig. 3). A small polylinker containing ClaI, BamHI and EcoRI restriction nuclease sites was introduced into the U3 region of the 3' LTR. The polylinker can be used to clone single promoter (H1) siRNA expression cassette or double promoter (U6/H1) siRNA expression cassette.

[0077] In one embodiment, a single promoter H1siRNA expression cassette was assembled from chemically synthesized oligonucleotides and cloned into the ClaI-BamHI sites to direct transcription of small hairpin RNAs, which are processed into functional siRNA by cellular enzymes. The resulting single promoter expression cassette pLSLP construct was very similar in design to other retroviral vectors that have been used successfully for cloning and expression of siRNA (see, e.g., Qin, X.F., et al. PNAS, 100:183-88 (2003); Tiscorna, G., et al., PNAS, 100:1844-48 (2003); and Xia, H., et al. Nature Biotech, 20:1006-10 (2002)).

[0078] Two exemplary effector constructs have been made and cloned into the pLSLP-H1siRNA vector (see Fig. 9). The constructs in Figure 9 differ in the length of the siRNA inserts. In one case, the siRNA component is 19 bp in length, in the other case, the siRNA is 27 bp in length. The duplex at the top of the Figure shows the sequence of the lentiviral vector after digestion with BamH1 and EcoRI. The dotted lines indicate the position of the removed nucleotides (a stuffer sequence

6,555,107

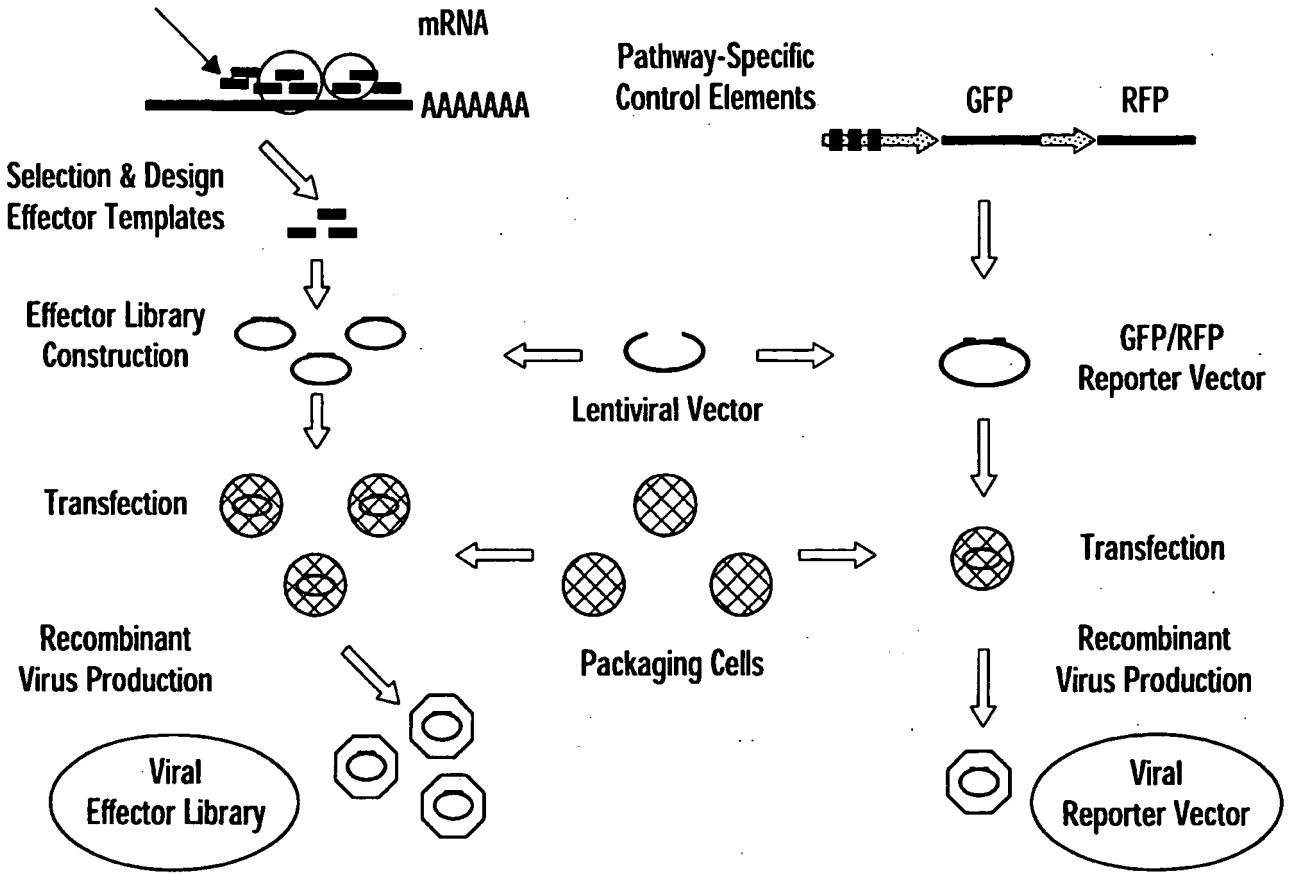


FIG. 6

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Title: REPORTER VECTORS FOR GENE FUNCTION ANALYSIS
Inventor: Chumakov et al.
Express Mail No.: EL 880273535 US Page 8 of 12

FIG. 8

Atty Dkt No.: SBIO/0002.D1
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Filed: Herewith
Title: REPORTER VECTORS FOR GENE FUNCTION ANALYSIS
Inventor: Chumakov et al.
Express Mail No.: EL 802273535 US Page 8 of 12

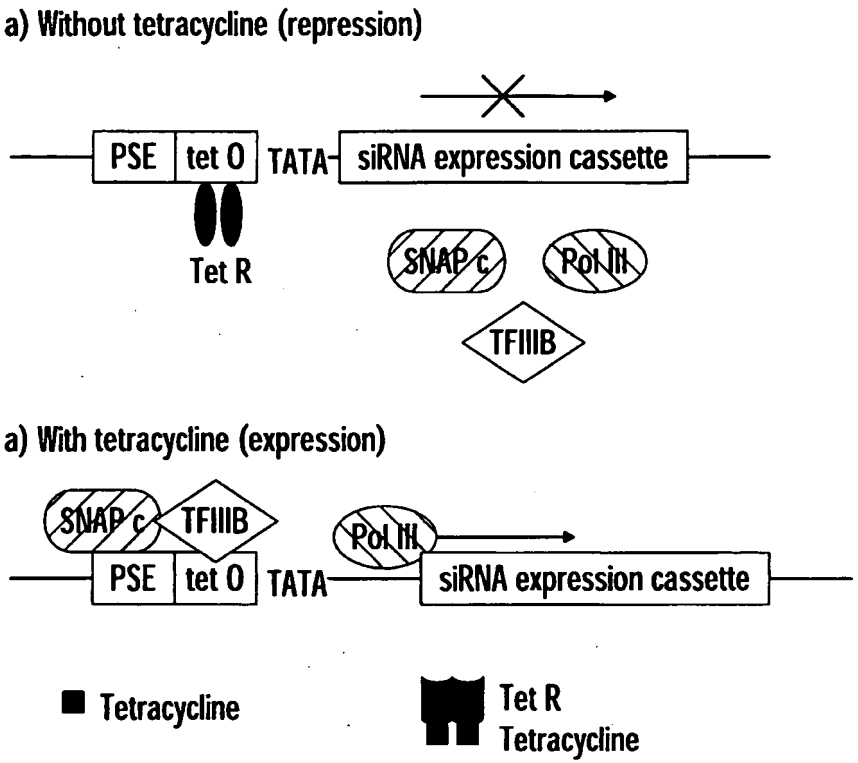

FIG. 11

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Title: REPORTER VECTORS FOR GENE FUNCTION ANALYSIS
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Lentivirus-delivered stable gene silencing by RNAi in primary cells

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[6]University of California, Los Angeles, AIDS Institute, David Geffen School of Medicine, Department of Microbiology, Immunology, Molecular Genetics & Medicine, Los Angeles, California 90025, USA

ABSTRACT

Genome-wide genetic approaches have proven useful for examining pathways of biological significance in model organisms such as *Saccharomyces cerevisiae, Drosophila melanogastor,* and *Caenorhabditis elegans,* but similar techniques have proven difficult to apply to mammalian systems. Although manipulation of the murine genome has led to identification of genes and their function, this approach is laborious, expensive, and often leads to lethal phenotypes. RNA interference (RNAi) is an evolutionarily conserved process of gene silencing that has become a powerful tool for investigating gene function by reverse genetics. Here we describe the delivery of cassettes expressing hairpin RNA targeting green fluorescent protein (GFP) using Moloney leukemia virus-based and lentivirus-based retroviral vectors. Both transformed cell lines and primary dendritic cells, normally refractory to transfection-based gene transfer, demonstrated stable silencing of targeted genes, including the tumor suppressor gene *TP53* in normal human fibroblasts. This report demonstrates that both Moloney leukemia virus and lentivirus vector-mediated expression of RNAi can achieve effective, stable gene silencing in diverse biological systems and will assist in elucidating gene functions in numerous cell types including primary cells.

Keywords: Retrovirus; hairpin RNA; siRNA; knockdown; dendritic cells

INTRODUCTION

Genetic analysis through targeted gene deletion in mammals has been limited principally to murine models. The discovery that the RNA interference (RNAi) pathway is active in mammals (Elbashir et al. 2001) raised the possibility that reverse genetic approaches using RNAi could be applied to mammalian systems. Like targeted gene deletion, RNAi-based silencing permits analysis of gene function in primary cells (McCaffrey et al. 2002; Novina et al. 2002). Compared to knockout genetics, RNAi-based silencing is rapid, cost effective, and can be easily adapted to study homologous gene function in a wide variety of organisms.

RNAi depends upon the formation of double-strand RNA (dsRNA) whose antisense strand is complementary to the transcript of a targeted gene. Two distinct steps are involved (for review see Hutvagner and Zamore 2002; McManus and Sharp 2002). In the first, the enzyme Dicer cleaves long dsRNA into short interfering RNA (siRNA) molecules of 21–23 bp in length. In the second, a multicomponent RNA-induced silencing complex (RISC) uses siRNA to guide the sequence-specific cleavage of the RNA transcripts of the target gene. Gene silencing by this mechanism has been achieved by transfection of chemically synthesized siRNA into host cells, by-passing the "dicing" step, to silence ("knockdown") gene expression (McManus and Sharp 2002).

Several recently developed (Brummelkamp et al. 2002a; McCaffrey et al. 2002; McManus et al. 2002; Paddison et al. 2002; Paul et al. 2002; Sui et al. 2002; Xia 2002; Yu et al.

[7]These authors contributed equally to this work.

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Article and publication are at http://www.rnajournal.org/cgi/doi/10.1261/rna.2192803.

2002) vector-based strategies that contain stem-loop constructs encoding hairpin RNAs lead to the intracellular generation of siRNA-like species. Dicer cleavage of hairpin RNA can generate small dsRNA that silence expression of genes whose transcripts are complementary to one of the two strands of the hairpin RNA (for review, see Paddison and Hannon 2002). However, most vector-based hairpin expression systems have demonstrated only transient knockdown of gene expression. More recently, knockdown of gene expression has been achieved using retroviral vector constructs that express hairpin RNAs within vector-infected cells and produce long-term knockdown within cultured cell lines (Barton et al. 2002; Brummelkamp et al. 2002b; Devroe and Silver 2002; Paddison and Hannon 2002).

To facilitate stable, long-term knockdown in populations of cells refractory to transfection-based gene transfer techniques, we designed a retroviral vector that permits delivery of stem-loop cassettes. We report the development of a versatile system of retrovirus-based vectors that make it possible to achieve durable, high efficiency siRNA-dependent gene silencing in a wide variety of cells.

RESULTS

Stable gene silencing in HeLa-GFP cells

Several studies have demonstrated that the cellular machinery required for RNAi exists in mammalian cells. To study gene function in primary mammalian cells in vitro using RNAi-based gene silencing technology, we designed a vector system (Fig. 1A) in which the RNA polymerase III (pol-III) promoter of the U6 small nuclear RNA gene (U6) was used to drive high levels of expression of a small hairpin RNA. An inverted repeat sequence (a stem-loop, sl) with stem sequences complementary to the mRNA specifying green fluorescent protein (GFP) was cloned 3′ of the U6 promoter and a five thymidine repeat sequence was added to serve as a strong pol-III termination signal (Gunnery et al. 1999), which results principally in hairpin RNA species containing four uridines. However, it is unclear from our analysis at which uridine transcription terminates. Because stable gene silencing is affected by retrovirus-delivered hairpin RNA, the vector system reported here is called Retrohair. Assuming that four uridines are incorporated into the transcript from the five thymidine pol-III transcription termination signal, Retrohair encodes a 52-nt hairpin RNA (Fig. 1B).

To ensure rapid reductions in protein levels in response to decreases in mRNA levels, we used an enhanced GFP protein with the PEST domain at the carboxy-terminus,

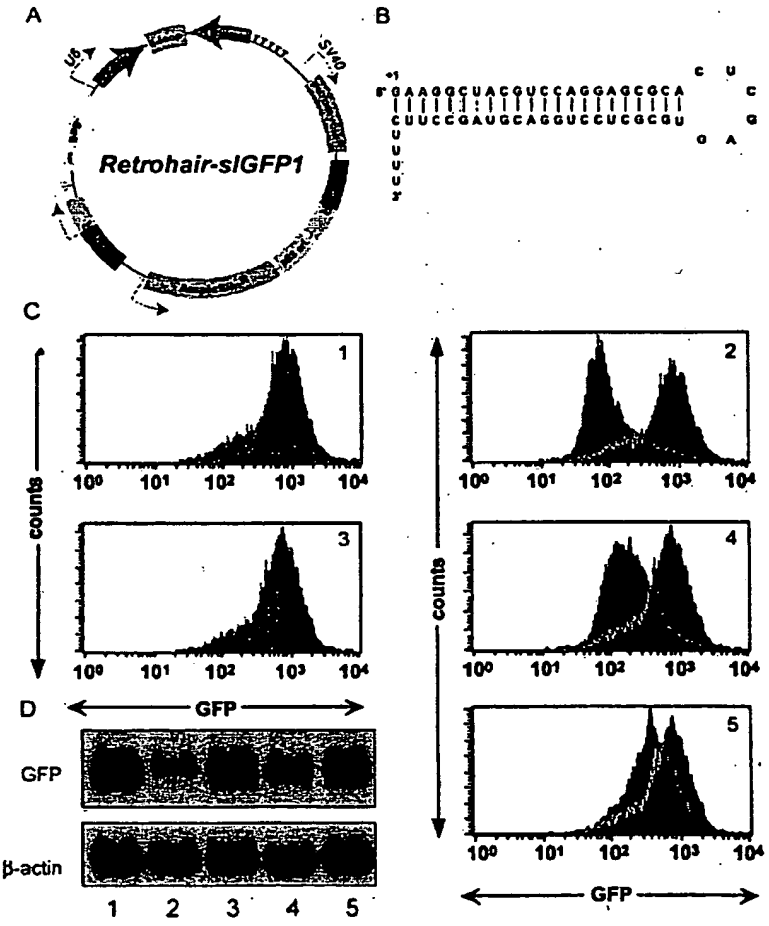

FIGURE 1. Sequence-specific knockdown of GFP expression by retrovirus-delivered GFP hairpin RNA expression. (*A*) Schematic presentation of Retrohair-slGFP, a replication incompetent retrovirus that contains a pol-III promoter (U6), a stem-loop cassette followed by a 5T pol-III termination signal, cloned 3′ to a truncated gag lacking a translation start sequence (ATG). Retrohair can be selected by addition of puromycin. (*B*) The stem-loop encoded hairpin RNA has sequence identity to a 21-nt region of GFP mRNA. (*C*) FACs analysis of GFP expression in HeLa-GFP infected with (*1* and *3*) empty vector controls, (*2*) Retrohair-slGFP1, (*4*) Retrohair-slGFP2, or (*5*) Retrohair-slGFP2mut. Overlay histograms in panels *2*, *4*, and *5* demonstrate the level of knockdown in cells infected with Retrohair-containing stem-loop cassettes (red) compared to cells infected with empty vector controls (green). (*D*) Northern blot analysis of GFP expression in HeLa-GFP cells described in *C* using a GFP-specific probe. Northern blot with a β-actin probe served as a loading control.

resulting in a fusion protein with a half-life of approximately 1 h. We developed a HeLa cell line that stably expresses this fusion protein (HeLa-GFP) to provide a sensitive, rapidly responding reporter system to examine gene silencing and its kinetics.

To examine the efficiency of retrovirus-delivered RNAi-based knockdown, HeLa-GFP were infected with virus expressing either wild-type or point mutant hairpin RNA sequences directed against GFP (Fig. 1C). Fluorescence-activated cell sorting (FACS) analysis of GFP expression in HeLa-GFP cells infected with wild-type Retrohair-slGFP1 demonstrated a 10-fold reduction in mean fluorescence intensity (MFI: 691 → 76) relative to empty vector controls (containing the U6 promoter but lacking slGFP1; Fig. 1C). In contrast, a construct containing a point mutation in the stem sequence (Retrohair-slGFP2mut) resulted in a twofold reduction in gene expression (MFI: 620 → 349). Northern blot analysis demonstrated decreased GFP mRNA levels only in the samples infected with wild-type viruses but not in mutant or empty vector controls (Fig. 1D). Taken together, these data indicate that the hairpin RNA-based knockdown was specific and that silencing correlated with decreased GFP mRNA levels. Direct comparison demonstrated that Retrohair-slGFP1 was more efficient at silencing than Retrohair-slGFP2 (MFI = 76 vs. 158). Therefore, we used the Retrohair-slGFP1 vector for subsequent studies.

To determine the stability of the retrovirus-delivered RNAi, we infected HeLa-GFP with the empty vector control or with Retrohair-slGFP1. After infection, cells were selected by the addition of puromycin and GFP expression was analyzed (Fig. 2). GFP expression was not reduced in cells infected with the empty vector control, but was markedly decreased in cells infected with Retrohair-slGFP1 (Fig. 2A). Following Retrohair-slGFP1 infection, the population exhibited a bimodal distribution and the overall MFI was 342 compared to the average MFI of the control population (859). Following 2 wk of selection, the population became more homogenous and the MFI decreased further to 89. Similar results were obtained after selection of cells infected with virus containing Retrohair-slGFP2 (data not shown).

To determine whether continuous selective pressure was necessary to maintain gene knockdown, puromycin was removed from the cells 2 wk after selection had been initiated. Analysis of GFP expression demonstrated that the MFIs were identical to the cells that were continuously selected (Fig. 2A), indicating that constant selective pressure was not

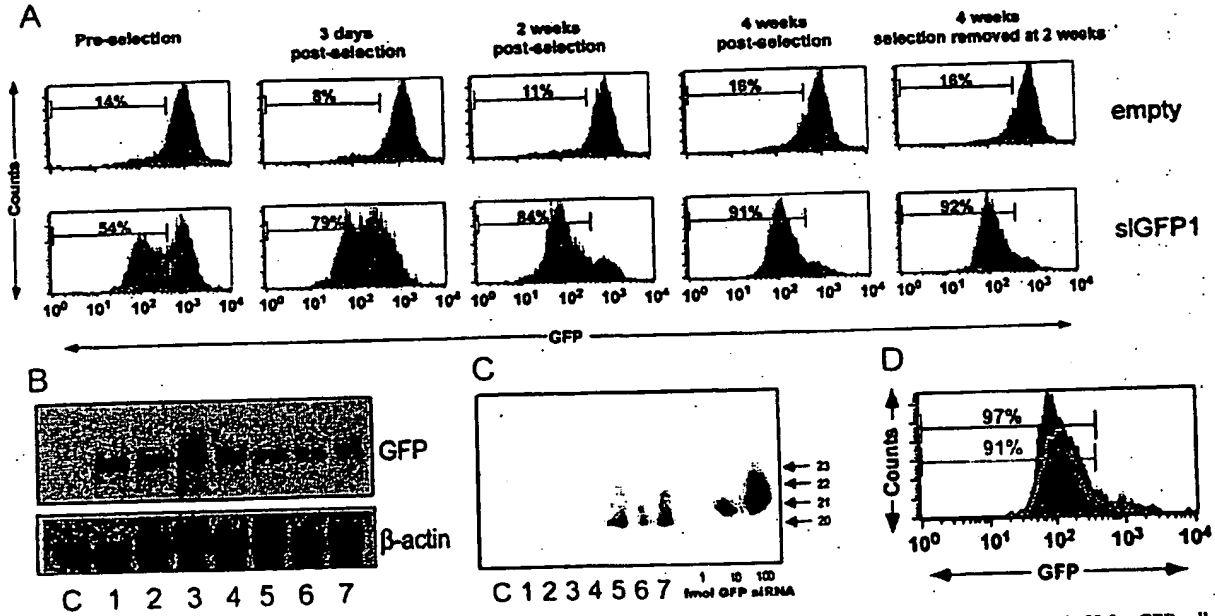

FIGURE 2. Time course of selection of GFP knockdown in RNAi-stable HeLa-GFP cells. (*A*) FACS analysis of GFP expression in HeLa-GFP cells infected with Retrohair-lacking a stem-loop (empty, *top* panels) or with Retrohair-slGFP1 (-slGFP1, *bottom* panels). GFP expression was measured in HeLa-GFP either preselection, with 3 d, 2 wk, or 4 wk of puromycin selection or in HeLa-GFP after 2 wk of growth with puromycin selection followed by 2 wk of growth without puromycin selection. (*B*) Northern blot analysis of GFP expression in HeLa (lane *C*), in HeLa-GFP infected with Retrohair at (lane *1*) 3 d (lane *2*) 2 wk, (lane *3*) 4 wk, or infected with Retrohair-slGFP1 at (lane *4*) 3 d (lane *5*) 2 wk, (lane *6*) 4 wk, or at (lane *7*) 2 wk with selection and 2 wk without puromycin selection. β-Actin expression served as a loading control. (*C*) Modified Northern blot analysis of the samples described in *B* using an RNA probe (sense strand) with complementarity to the antisense strand of the GFP hairpin RNA. Samples were normalized by total RNA content. Titration of GFP siRNA was used as a size marker and for quantitation by densitometry of small RNAs. (*D*) FACS analysis of GFP expression in HeLa-GFP infected with Retrohair-slGFP1 4 wk after selection on puromycin (green) or infected with Retrohair-slGFP1 4 wk after selection on puromycin and then superinfected with Retrohair-slGFP2 (red).

required to maintain hairpin RNA expression and silencing of GFP expression for 4 wk. Northern blot analysis of these cells revealed that loss of GFP expression correlated with loss of GFP mRNA (Fig. 2B). Although constant selective pressure may not be needed to maintain silencing of genes with a neutral or growth-promoting phenotype, it is possible that continuous selective pressure may be required for stable silencing of genes whose loss have a negative effect on cell growth or are essential for cell survival.

RNAi is mediated by short, 21–23-bp dsRNA. Processing of the GFP1 hairpin RNA into GFP1 siRNA was demonstrated by the presence of multiple small RNA products in modified Northern blot analysis (Fig. 2C). The major RNA cleavage product comigrated with a 20-nt species and minor products comigrated with 21-, 22-, and 23-nt species. In this analysis, 11.5 μg total RNA corresponding to approximately 7.6×10^5 cell equivalents was used (Fig. 2C). If densitometry signals from each cleavage product are added, approximately 8 fmoles of GFP siRNA were present in cells following 2 wk of selection. As a conservative estimate of silencing (assuming that the approximately 85% of the HeLa-GFP that were actively silencing GFP expression were responsible for hairpin RNA expression), an estimated 8.6×10^{-21} moles siRNA per cell or approximately 5×10^3 molecules of siRNA derived from hairpin RNA precursors were associated with each of the actively silencing HeLa-GFP. It is not known which of the cleavage product(s) is the active species.

We noted that a small percentage of the HeLa-GFP remained GFP-positive following several weeks of selection. Superinfection of these cells with Retrohair-slGFP2 resulted in further reduction of GFP expression in already actively silencing HeLa-GFP previously infected with Retrohair-slGFP1 (Fig. 2D). These results suggest that cells containing high levels of GFP are not inherently resistant to RNAi and that some GFP-positive cells were infected (because they were puromycin resistant) but expressed insufficient siRNA for GFP silencing. Taken together, these studies indicate that (1) infection of HeLa-GFP with Retrohair-slGFP1 reduced GFP gene expression, (2) selection enriched for HeLa-GFP with higher expression levels of the hairpin RNA, and (3) continuous expression of the GFP hairpin RNAs and maintenance of the knockdown phenotype did not require continuous selective pressure.

Gene silencing in primary dendritic cells

To extend these studies to primary mammalian cells that are refractory to gene transfer (Esslinger et al. 2002), we turned to dendritic cells (DC). DC are the most potent antigen-presenting cells (APC) known. Capable of activating naïve B and T cells, they play an important role in initiating host immune responses (Steinman and Pope 2002). DC also play a critical role in maintenance of tolerance (Steinman and Nussenzweig 2002). Thus, DC are under intensive study for their potential therapeutic uses. To determine if these cells are susceptible to retrovirus-delivered siRNA-directed gene silencing, we examined bone marrow-derived (BM) DC from a GFP transgenic mouse infected ex vivo with Retrohair-slGFP1 vector or an empty vector. GFP fluorescence microscopy indicated that GFP expression was lower in cells infected with the slGFP1-containing vector than in cells infected with the empty vector control (Fig. 3A). Consistent with this observation, 33% of DC-GFP infected with Retrohair-slGFP1 were observed in the GFP-negative gate compared to 11% of DC-GFP infected with the empty vector control (Fig. 3B, panels 1 and 2). The DC-GFP population demonstrates a wide range of GFP expression. Furthermore, the MFI of the entire population of Retrohair-slGFP1-infected DC-GFP was about 80% lower than that of the control vector-infected DC-GFP population (Fig. 3B, panel 3). The overall reduction in MFI suggested that the majority of DC-GFP had been infected by the Retrohair-slGFP1 virus. It may well be that the cells with the greatest reduction in fluorescence contained the highest level of hairpin RNA expression. High levels of hairpin RNA expression may be due to the favorable sites of proviral integration and/or to the number of proviral integration sites per cell.

Primary cells have been shown to possess the machinery to silence gene expression using siRNA (McCaffrey et al. 2002; Novina et al. 2002). Our data show that the hairpin is processed indicating that primary cells contain the RNAi machinery required to generate siRNA from the hairpin RNA (Fig. 3C). Similar to the HeLa-GFP experiment, several small processed RNA species with relative mobility of oligonucleotides 20–23 bp in length were observed in DC-GFP by modified Northern blot analysis (Fig. 3C). In this experiment, 1.65 μg total RNA derived from 2×10^6 infected DC was analyzed. Densitometry signals from each of the Retrohair-slGFP1 cleavage products were added and amounted to 5 fmoles of GFP-siRNA. As a conservative estimate of silencing (assuming that 33% of the DC actively silencing GFP expression were responsible for hairpin RNA expression), an estimated 7.5×10^{-21} moles siRNA per cell or approximately 4.5×10^3 molecules of siRNA were derived from hairpin RNA precursors in each actively silencing DC. This value is in agreement with the estimated number of siRNA molecules in GFP-silencing HeLa-GFP (see above).

Lentiviral delivery of stable gene silencing

Moloney-based vector systems permit gene transduction of proliferating cells but are incapable of productively infecting nondividing cells, limiting its usefulness in primary cells both in vitro and in vivo. Therefore, we constructed a lentiviral vector containing the U6 driven stem-loop directed

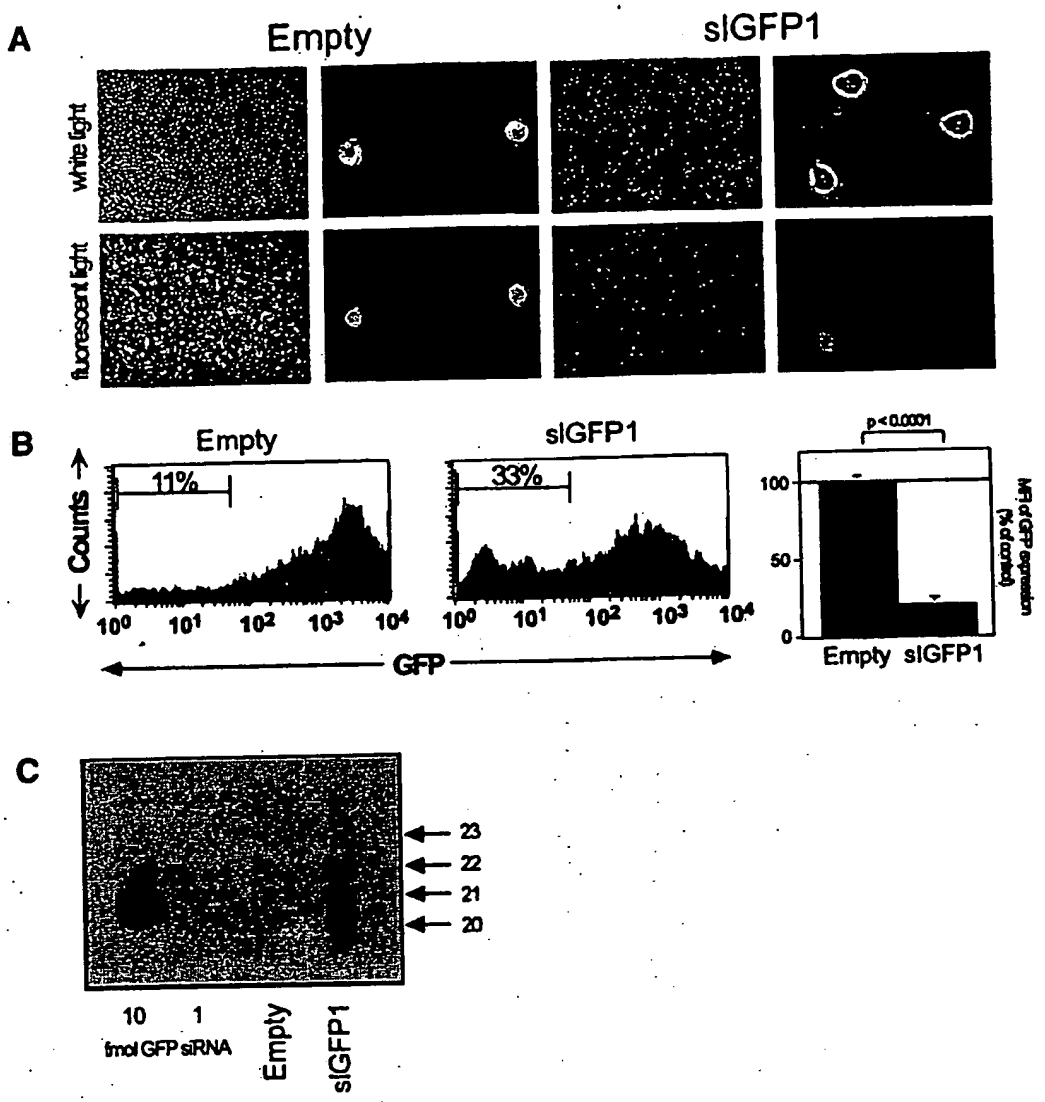

FIGURE 3. Knockdown of GFP expression in DC-GFP. (*A*) Photomicroscopy of DC-GFP visualized by white light or by fluorescent light after infection with Retrohair or with Retrohair-slGFP1(slGFP1). (*B*) FACS of GFP expression in DC-GFP infected with the Retrohair or Retrohair-slGFP1 retrovirus. A bar graph depicts the percent decrease in GFP expression as measured by MFI. Error bars represent the average (±*SD*) of six experiments. (*C*) Modified Northern blot analysis of Retrohair and Retrohair-slGFP1-infected DC-GFP using an RNA probe (sense strand) with complementarity to the antisense strand of the GFP hairpin RNA. Samples were normalized by total RNA content. Titration of GFP siRNA was used as size marker and for quantitation by densitometry of small RNAs.

against GFP. Because stable gene silencing is affected by lentivirus-delivered hairpin RNA, the vector system is called Lentihair (Fig. 4A). HeLa-GFP were infected with Lentihair-slGFP1 and GFP expression was analyzed by FACS 3 d later (Fig. 4B). GFP expression was reduced more than 10-fold relative to nonspecific stem-loop lentiviral control infections.

Primary DC-GFP were also infected with Lentihair-slGFP1 and resulted in a greater knockdown of GFP than was observed with Retrohair-slGFP1, 50% knockdown versus 33% (Fig. 4C). GFP expression in DC-GFP was reduced (MFI: 1174 → 87) in cells infected with Lentihair-slGFP1 compared to uninfected controls. We also noted that there was a modest knockdown of GFP expression (>3-fold) in DC-GFP infected with a lentivirus that targeted luciferase (Lentihair-slLuc), suggesting that there is some nonspecific knockdown in these cells. Interestingly, the lentivirus-delivered RNAi was more efficient in both HeLa-GFP and DC-

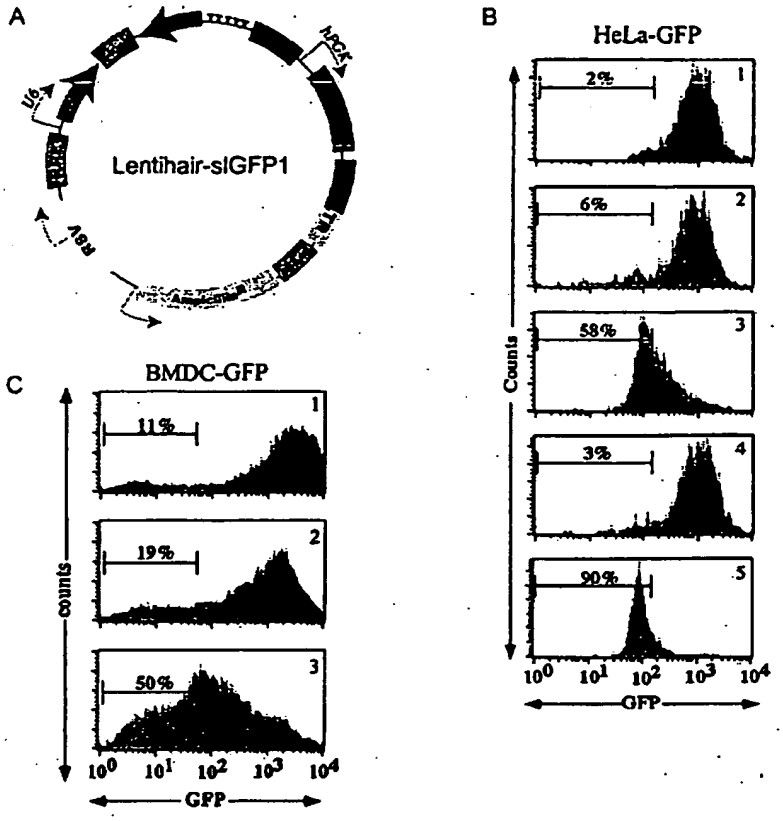

FIGURE 4. Knockdown of GFP expression by lentivirus-delivered RNAi. (*A*) Schematic presentation of the lentiviral construct. Lentihair is a replication incompetent, self-inactivating retrovirus generated by cloning the U6 promoter and stem-loop cassette targeted to luciferase (Lentihair-slLUC) or GFP (Lentihair-slGFP1) 5′ of the cPPT. A puromycin resistance gene was cloned 3′ of the human hPGK promoter. (*B*) FACS analysis of GFP expression in HeLa-GFP cells (*1*) uninfected or infected with (*2*) Retrohair, (*3*) Retrohair-slGFP1, (*4*) Lentihair-slLUC or (*5*) Lentihair-slGFP. (*C*) FACS analysis of GFP expression in (*1*) uninfected, (*2*) Lentihair-slLUC, or (*3*) Lentihair-slGFP infected DC-GFP.

GFP compared to Moloney leukemia virus-delivered RNAi. The Moloney-based vector contains a transcriptionally active LTR whereas the lentivirus is transcriptionally inactive due to mutations in the U3 region of the LTR. Therefore, differences between Moloney- and lentivirus-delivered gene silencing may be due to different vector configurations.

DISCUSSION

Utilizing several biological systems, we demonstrate retroviral delivery and intracellular processing of hairpin RNA in a variety of cell types, including primary mammalian cells. We have extended the analysis of retrovirus-delivered stable gene silencing to normal human cells, and the vector system reported was used to silence medically relevant genes such as p53 (data can be accessed at www2.dfci.harvard.edu/hahnlab). This data demonstrates that a complete knockout is not required to produce a biological phenomenon. By understanding and using retrovirus-delivered knockdown in primary cells, the function of human genes in biological and disease processes may be determined.

Although retroviral delivery of hairpin RNA has been shown to result in the knockdown of targeted proteins in human primary fibroblasts (Barton and Medzhitov 2002), we have demonstrated hairpin RNA processing into siRNA in bone marrow-derived primary DC. The level of RNA hairpin expression and its processing into siRNAs in a target cell will likely determine the extent of gene knockdown. It is possible that different cell types vary in their ability to process long dsRNA and hairpin RNA into smaller cleavage products. We found that HeLa-GFP and DC-GFP contained similar levels of processed RNAs. The slightly higher levels of small RNAs found in HeLa-GFP may be due to higher levels of hairpin RNA processing or, alternatively, to the amount of hairpin RNA expressed from the integrated provirus(es). In the latter instance, superinfection of cells already infected with a virus encoding one stem-loop with another virus having either the same sequence or a sequence directed against a different region of the same gene may yield greater knockdown of that gene. Indeed, we observed that superinfection of HeLa-GFP resulted in greater knockdown, suggesting that gene expression can be further silenced by retroviral vectors targeting different sequences in the same mRNA.

DC are critical for initiating primary immune responses (Stark et al. 1998; Jung et al. 2002). To initiate a primary immune response, the DC undergo a series of maturation steps ultimately producing a fully differentiated immunocompetent cell poised to function as an activated APC. Efforts to genetically manipulate these cells (without activating them) by conventional transfection methods have proven ineffectual (Gasperi et al. 1999; Esslinger et al. 2002). Adeno-vectors are efficient gene transducers (Song et al. 1997; Klein et al. 2000) but they activate immune responses (Marshall 1999). Moreover, preexisting immunity to adenoviral vectors (Yang et al. 1994) potentially limits their use in vivo. Previous studies have indicated that lentiviral infection of DC does not activate these cells (Esslinger et al. 2002) raising the possibility that stable gene

silencing by retroviral vector delivery may be able to modulate host immune responses. Lentihair-slGFP1 infection of HeLa-GFP and DC-GFP indicates that this vector configuration is highly effective in gene silencing.

The principal advantage of the lentiviral system is that it will allow gene silencing in nondividing cells and will therefore expand the usefulness of the RNAi-based gene silencing system. It is interesting to note that we observed some nonspecific knockdown in DC-GFP when we used Lentihair-slLUC, an effect not observed in HeLa-GFP. The nonspecific knockdown in DC-GFP may be due to activation of the interferon pathway in these cells. Recognition of dsRNA by DC result in their maturation and the DC-GFP may have demonstrated some degree of maturation following lentivirus infection (Le Bon and Tough 2002). Preliminary data suggest that limiting lentiviral infection of DC to one infection rather than two results in fewer cells exhibiting knockdown (32% vs. 50%). In addition, nonspecific knockdown with the Lentihair-slLUC was reduced to twofold. Therefore, changing the kinetics or dose of infection may limit the nonspecific knockdown. Our data indicate that the system for retrovirus delivery of RNAi-based gene silencing can be adapted to use in lentiviral systems, thereby opening opportunities to analyze gene function in diverse cells, dividing or not, under physiological conditions.

RNAi provides plants with effective antiviral defenses (for review, see Waterhouse et al. 2001). A natural role for mammalian RNAi in antiviral defenses has not been shown. It is possible that RNAi-like antiviral defenses may assist the host immune response against viral infection, including the retroviral vector used to deliver the stem-loop-containing cassette. However, our data indicate that productive infection can occur and that the retroviral RNA can be produced and packaged, and is unlikely to be targeted for degradation by an RNAi-like mechanism despite the presence of complementary sequences in the provirus. Thus, our system demonstrates a proof of principle that retroviral delivery of RNAi can be used for the elucidation of gene function in a wide variety of cell types, including differentiated, nondividing cells.

MATERIALS AND METHODS

Vector design

The U6 promoter (a gift from Nouria Hernandez, Cold Spring Harbor Laboratoy, Cold Spring Harbor, New York) was cloned into pBABEpuro (pBp) with the addition of an *ApaI* site at the transcription start site (Morgenstern and Land 1990). The pBpU6 was digested with *ApaI*, blunted, and digested with *Eco*RI. The following oligomers were used: GFP1 forward 5'P-GGCTACGTC CAGGAGCGCACCCTCGAGGGTGCGCTCCTGGACGTAGCCT TTTTG-3' and GFP1 reverse 5'P-AATTCAAAAAGGCTACGTC CAGGGCGACCCTCGAGGGTGCGCTCCTGGACGGAGCC-3' and GFP2 forward 5'P-CGCAAGCTGACCCTGAGTTCATTCAA GAGATGAACTTCAGGGTCAGCTTGCTTTTTG-3' and GFP2 back 5'P-AATTCAAAAAGCAAGCTGACCCTGAAGTTCATCT CTTGAATGAACTTCAGGGTCAGCTTGCGGGCC-3'. In the process of screening GFP2 clones, one contained a point mutation (mutation is underscored: 5'-CGCAAGCTGACCCTGAAGTT TATTCAAGAGATGAACTTCAGGGTCAGCTTGCTTTTTG-3').

The Lentihair-slLUC lentiviral construct contains the U6 promoter and a stem-loop directed against the luciferase gene (LUC; Paddison et al. 2002) or GFP cloned 5' of the central polypurine tract (cPPT) from pRRL-cPPT-hPGKEsin (Dull et al. 1998). A cPPT was inserted 5' of the human phosphoglycerate kinase promoter in the pRRL-hPGKsin vector. The puromycin-resistant gene was cloned 3' of the human phosphoglycerate kinase (hPGK) promoter.

Cell culture

HeLa cells, grown in Dulbecco Modified Eagle medium (DME) plus 10% heat-inactivated fetal calf serum (FCS) containing penicillin and streptomycin (pen/strep), were transfected with pd1EGFP-N1 (Clontech), selected with 500 μg/mL G418, and single-cell cloned.

Isolation, culture, and infection of BM-DC

Isolation, culture, and infection of BM-DC has been described (Inaba et al. 1992). Bone marrow was flushed from the femur and tibia of C57BL/6-EGFP mice (Dr. Okabe, Osaka University; Okabe et al. 1997), red blood cells were lysed, and the remaining cells were plated in RPMI1640 (plus 10% FCS, 2 mM L-glutamine, 10 mM HEPES, 50 μM 2-mercaptoethanol and pen/strep) at 1×10^6 cells/mL/well. The medium was supplemented (1:25) with culture supernatant from J5 cells expressing GM-CSF.

Viral production and infections

The pBp-amphotrophic viruses were produced by cotransfection of 293T cells with the pBp constructs and pCL10A1. The pBp-VSV-G viruses were produced by cotransfecting pBp constructs, pUMVC3, and pCMV-VSV-G (at a 9:1 ratio). Lentihair-slLUC and Lentihair-slGFP1 were produced by cotransfection with pHCMVG (Burns et al. 1993) and pCMVΔR8.20vpr (An et al. 1999). Transfections were carried out using Fugene 6 (Roche). Virus was harvested at 48 and 72 h posttransfection and infections were carried out in the presence of 10 μg/mL of polybrene and 10 mM HEPES. Following transduction, cells were selected with 1 μg/mL puromycin. DC were spin infected on days 1 and 2 at 2.5K for 1.5 h. Virus-containing supernatant was removed after 2 h.

Flow cytometry and microscopy

Flow cytometry was carried out using FACScalibur and Cellquest software. For imaging, DC were mounted onto microscope slides and images were collected on an Axioplan 2 microscope (Zeiss) using Axiovision Viewer 3 software (Zeiss).

Northern blot

Total RNA was prepared using the RNeasy mini kit. Northern blot was performed using the Northern Max Northern blotting kit. The

GFP probe was prepared by PCR amplification of a 497-nt fragment from pd1EGFP-N1 (5'-CCTACGGCAAGCTGACCCTGA AGTTCA-3' [forward] and 5'-GGACTGGGTGCTCAGGTAGTG GTTGT-3' [reverse]) that was purified using the Qiaquick gel extraction kit (Qiagen). The purified GFP fragment and the β-actin control (DECA template mouse β-actin control DNA) were labeled by random prime labeling using the Decaprime II DNA labeling kit (Ambion).

Modified Northern blot

Total RNA was prepared by extraction with Trizol (Molecular Research Center, Inc.) as recommended by the manufacturer. The resulting homogenate was centrifuged for 15 min at 20,000*g* (4°C), precipitated, and reextracted as above. The DC went through one round of extraction. The precipitated RNA pellets were washed in 70% ethanol, air-dried, and resuspended in nuclease-free H_2O. A total of 11.5 μg HeLa-GFP total RNA or 1.65 μg DC total RNA were run on a 15% sequencing gel, electroblotted to Hybond N membrane (Amersham), UV cross-linked and prehybridized overnight at 37°C in 5× SSPE, 2× Denhardt's solution, and 0.1× SDS containing 0.1 mg/mL denatured salmon sperm DNA and 0.1 mg/mL denatured yeast tRNA. The sense strand (100 pmoles) of GFP1 siRNA (5'P-GGCUACGUCCAGGAGCGCACC-3') was end labeled with polynucleotide kinase and [γ^{32}P]-ATP (150 μCi), purified on a G-25 MicroSpin Column (Amersham), heated for 5 min to 65°C, added to the prehybridization buffer, and hybridized overnight. Blots were washed at room temperature 2 × 5 min in 2× SSC and 0.1% SDS, 3 × 10 min in 0.1× SSC and 0.1% SDS and exposed to film.

ACKNOWLEDGMENTS

We thank Helen Cargill for assistance in preparation of the figures, Dr. Herman Eisen for the critical reading of the manuscript, and members of the WI-MIT-Dana-Farber RNAi Consortium for encouragement. We thank Dr. Hernandez for the U6 promoter. This work was supported by NCI F32 CA93033-01 (S.A.S.), NCI R01CA78461-04, Temin Award K01CA94223 (W.C.H.), NIH F32 AI10523 (C.D.N.), NIH MERIT grant R37-GM34277, R01-AI32486, NCI grant P01-CA42063, P30-CA14051 (P.A.S.), and NCI Cancer Center Support (Core) grant P30-CA14051 (P.A.S.) and grants from Merck/MIT and Co, Inc. (R.A.W.), a Doris Duke Charitable Foundation Clinical Scientist Development Award (W.C.H.), and a Kimmel Scholar Award (W.C.H.), and a Stressgen postdoctoral fellowship (D.P.). The work was supported by the Fidelity Investments Foundation (D.M.S.).

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Received December 2, 2002; accepted January 9, 2003.

REFERENCES

An, D.S., Morizono, K., Li, Q.X., Mao, S.H., Lu, S., and Chen, I.S. 1999. An inducible human immunodeficiency virus type 1 (HIV-1) vector which effectively suppresses HIV-1 replication. *J. Virol.* 73: 7671–7677.

Barton, G.M. and Medzhitov, R. 2002. Retroviral delivery of small interfering RNA into primary cells. *Proc. Natl. Acad. Sci.* 99: 14943–14945.

Brummelkamp, T.R., Bernards, R., and Agami, R. 2002a. A system for stable expression of short interfering RNAs in mammalian cells. *Science* 296: 550–553.

———. 2002b. Stable suppression of tumorigenicity by virus-mediated RNA interference. *Cancer Cell.* Published online.

Burns, J.C., Friedmann, T., Driever, W., Burrascano, M., and Yee, J.K. 1993. Vesicular stomatitis virus C glycoprotein pseudotyped retroviral vectors: concentration to very high titer and efficient gene transfer into mammalian and nonmammalian cells. *Proc. Natl. Acad. Sci.* 90: 8033–8037.

Devroe, E. and Silver, P.A. 2002. Retrovirus-delivered siRNA. *BMC Biotechnol.* 2: 15.

Dull, T., Zufferey, R., Kelly, M., Mandel, R.J., Nguyen, M., Trono, D., and Naldini, L. 1998. A third-generation lentivirus vector with a conditional packaging system. *J. Virol.* 72: 8463–8471.

Elbashir, S.M., Harborth, J., Lendeckel, W., Yalcin, A., Weber, K., and Tuschl, T. 2001. Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature* 411: 494–498.

Esslinger, C., Romero, P., and MacDonald, H.R. 2002. Efficient transduction of dendritic cells and induction of a T-cell response by third-generation lentivectors. *Hum. Gene Ther.* 13: 1091–1100.

Gasperi, C., Rescigno, M., Granucci, F., Citterio, S., Matyszak, M.K., Sciurpi, M.T., Lanfrancone, L., and Ricciardi-Gastagnoli, P. 1999. Retroviral gene transfer, rapid selection, and maintenance of the immature phenotype in mouse dendritic cells. *J. Leukoc. Biol.* 66: 263–267.

Gunnery, S., Ma, Y., and Mathers, M.B. 1999. Termination sequence requirements vary among genes transcribed by RNA polymerase III. *J. Mol. Biol.* 286: 745–757.

Hutvagner, G. and Zamore, P.D. 2002. RNAi: Nature abhors a double-strand. *Curr. Opin. Genet. Dev.* 12: 225–232.

Inaba, K., Inaba, M., Romani, N., Aya, H., Deguchi, M., Ikehara, S., Muramatsu, S., and Steinman, R.M. 1992. Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor. *J. Exp. Med.* 176: 1693–1702.

Jung, S., Unutmaz, D., Wong, P., Sano, G., De los Santos, K., Sparwasser, T., Wu, S., Vuthoori, S., Ko, K., Zavala, F., et al. 2002. In vivo depletion of CD11c(+) dendritic cells abrogates priming of CD8(+) T cells by exogenous cell-associated antigens. *Immunity* 17: 211.

Klein, C., Bueler, H., and Mulligan, R.C. 2000. Comparative analysis of genetically modified dendritic cells and tumor cells as therapeutic cancer vaccines. *J. Exp. Med.* 191: 1699–1708.

Le Bon, A. and Tough, D.F. 2002. Links between innate and adaptive immunity via type I interferon. *Curr. Opin. Immunol.* 14: 432–436.

Marshall, E. 1999. Gene therapy death prompts review of adenovirus vector. *Science* 286: 2244–2245.

McCaffrey, A.P., Meuse, L., Pham, T.T., Conklin, D.S., Hannon, G.J., and Kay, M.A. 2002. RNA interference in adult mice. *Nature* 418: 38–39.

McManus, M.T. and Sharp, P.A. 2002. Gene silencing in mammals by small interfering RNAs. *Nat. Rev. Genet.* 3: 737–747.

McManus, M.T., Petersen, C.P., Haines, B.B., Chen, J., and Sharp, P.A. 2002. Gene silencing using micro-RNA designed hairpins. *RNA* 8: 842–850.

Morgenstern, J.P. and Land, H. 1990. Advanced mammalian gene transfer: High titre retroviral vectors with multiple drug selection markers and a complementary helper-free packaging cell line. *Nucleic Acids Res.* 18: 3587–3596.

Novina, C.D., Murray, M.F., Dykxhoorn, D.M., Beresford, P.J., Riess, J., Lee, S.K., Collman, R.G., Lieberman, J., Shankar, P., and Sharp, P.A. 2002. siRNA-directed inhibition of HIV-1 infection. *Nat. Med.* 8: 681–686.

Okabe, M., Ikawa, M., Kominami, K., Nakanishi, T., and Nishimune, Y. 1997. "Green mice" as a source of ubiquitous green cells. *FEBS Lett.* 407: 313–319.

Paddison, P.J. and Hannon, G.J. 2002. RNA interference: The new somatic cell genetics? *Cancer Cell* 2: 17–23.

Paddison, P.J., Caudy, A.A., Bernstein, E., Hannon, G.J., and Conklin, D.S. 2002. Short hairpin RNAs (shRNAs) induce sequence-specific silencing in mammalian cells. *Genes & Dev.* 16: 948–958.

Paul, C.P., Good, P.D., Winer, I., and Engelke, D.R. 2002. Effective expression of small interfering RNA in human cells. *Nat. Biotechnol.* 20: 505–508.

Song, W., Kong, H.L., Carpenter, H., Torii, H., Granstein, R., Rafii, S., Moore, M.A., and Crystal, R.G. 1997. Dendritic cells genetically modified with an adenovirus vector encoding the cDNA for a model antigen induce protective and therapeutic antitumor immunity. *J. Exp. Med.* 186: 1247–1256.

Stark, G.R., Kerr, I.M., Williams, B.R., Silverman, R.H., and Schreiber, R.D. 1998. How cells respond to interferons. *Annu. Rev. Biochem.* 67: 227–264.

Steinman, R.M. and Nussenzweig, M.C. 2002. Avoiding horror autotoxicus: The importance of dendritic cells in peripheral T cell tolerance. *Proc. Natl. Acad. Sci.* 99: 351–358.

Steinman, R.M. and Pope, M. 2002. Exploiting dendritic cells to improve vaccine efficacy. *J. Clin. Invest.* 109: 1519–1526.

Sui, G., Soohoo, C., Affar, el B., Gay, F., Shi, Y., and Forrester, W.C. 2002. A DNA vector-based RNAi technology to suppress gene expression in mammalian cells. *Proc. Natl. Acad. Sci.* 99: 5515–5520.

Waterhouse, P.M., Wang, M.B., and Lough, T. 2001. Gene silencing as an adaptive defence against viruses. *Nature* 411: 834–842.

Xia, H., Mao, Q., Paulson, H.L., and Davidson, B.L. 2002. siRNA-mediated gene silencing in vitro and in vivo. *Nat. Biotechnol.* 20: 1006–1010.

Yang, Y., Nunes, F.A., Berencsi, K., Furth, E.E., Gonczol, E., and Wilson, J.M. 1994. Cellular immunity to viral antigens limits E1-deleted adenoviruses for gene therapy. *Proc. Natl. Acad. Sci.* 91: 4407–4411.

Yu, J.Y., DeRuiter, S.L., and Turner, D.L. 2002. RNA interference by expression of short-interfering RNAs and hairpin RNAs in mammalian cells. *Proc. Natl. Acad. Sci.* 99: 6047–6052.

Inhibiting HIV-1 infection in human T cells by lentiviral-mediated delivery of small interfering RNA against CCR5

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Contributed by David Baltimore, November 11, 2002

Double-stranded RNAs ~21 nucleotides long [small interfering RNA (siRNA)] are recognized as powerful reagents to reduce the expression of specific genes. To use them as reagents to protect cells against viral infection, effective methods for introducing siRNAs into primary cells are required. Here, we describe success in constructing a lentivirus-based vector to introduce siRNAs against the HIV-1 coreceptor, CCR5, into human peripheral blood T lymphocytes. With high-titer vector stocks, >40% of the peripheral blood T lymphocytes could be transduced, and the expression of a potent CCR5-siRNA resulted in up to 10-fold inhibition of CCR5 expression on the cell surface over a period of 2 weeks in the absence of selection. In contrast, the expression of another major HIV-1 coreceptor, CXCR4, was not affected. Importantly, blocking CCR5 expression by siRNAs provided a substantial protection for the lymphocyte populations from CCR5-tropic HIV-1 virus infection, dropping infected cells by 3- to 7-fold; only a minimal effect on infection by a CXCR4-tropic virus was observed. Thus, our studies demonstrate the feasibility and potential of lentiviral vector-mediated delivery of siRNAs as a general means of intracellular immunization for the treatment of HIV-1 and other viral diseases.

Although the idea of protecting cells against HIV-1 infection by the internal production of a protective molecule ("intracellular immunization") was suggested 14 years ago (1), it has not been realized as a clinical procedure, partly because no macromolecule has proved potent enough and because of limitations in gene delivery vehicles. Recently, it has been appreciated that small, double-stranded RNAs (siRNA) can be powerful sequence-specific catalysts for targeted RNA destruction by means of an evolutionarily conserved mechanism known as RNA interference (RNAi) (2–5). The properties of siRNA suggest its potential as an "intracellular immunogen." With the discovery that siRNAs can be effectively produced as hairpin transcripts from RNA polymerase III (Pol III) promoters (6–11), intracellular synthesis of siRNAs has become feasible. The best vector for delivering an siRNA template would be a lentivirus vector derived from HIV-1, because such vectors stably infect nondividing cells and are not subject to the silencing imposed on other retrovirus vectors (a "turning the tables" approach; refs. 12 and 13). Furthermore, lentiviral vectors have proven to be effective in expressing transgenes within multiple lineages over prolonged periods of time and safe in SCID-hu and non-human primate hematopoietic stem cell transplants (14, 15). HIV-1 infection could be prevented by either an siRNA directed against viral RNA, as has been done in several *in vitro* models (16–20), or by targeting the mRNA for the primary HIV-1 coreceptor, CCR5. CCR5 suggests itself as a target because people who lack both genes for CCR5 (CCR5Δ32 homozygotes) are resistant to HIV-1 infection but are otherwise apparently normal (21–24). We report here the successful use of this approach.

Materials and Methods

Vector Construction. A human U6-RNA pol III promoter (−328 to +1) was amplified from HEK-293 genomic DNA with primers 5′-gggaattcccccagtggaaagacgcgcag-3′ and 5′-cggaagcttgaagaccacg-gtgtttcgtcctttccacaa-3′, in which a *Bbs*I site was introduced at the 3′ end allowing the insertion of siRNA sequences at the +1 position of the U6 transcript. The PCR fragment was cloned at *Eco*RI–*Hin*dIII sites of pBS-SKII plasmid (Stratagene). Sequencing analysis showed all of the individual clones recovered had a GCGCG insertion at the −267 position comparing to the published human U6-RNA promoter sequence (GenBank accession nos. X07425 and M14486). This difference might originate either from a PCR error or a natural polymorphism. Nevertheless, transient transfection assays showed that this promoter fragment was fully functional (X.-F.Q. and D.B., unpublished data). To construct the hairpin siRNA expression cassette, two complementary DNA oligos (see below) were synthesized, annealed, and inserted between *Bbs*I and *Xho*I sites immediately downstream of the U6 promoter: 5′- acc-g(n)18ttcaagaga(n)18ctttttc-3′; 3′-(n)18aagttctct(n)18gaaaaagagct-5′. The 19-nt sense and reverse complementary targeting sequences are highlighted in bold. Note that the sense targeting sequence always starts with G at position 1 (thus, the reverse complementary sequence ends with C), as required for the efficient transcription initiation from the U6 promoter (25). The siRNA cassette also features a TTCAAGAGA loop situated between the sense and reverse complementary targeting sequences and a TTTTT terminator at the 3′ end. The CCR5-siRNA (186) contains the sense targeting sequence of gagcatgactgacatctac corresponding to the 186–204 nucleotide positions of human CCR5 coding sequence (GenBank accession no. U57840), whereas the CCR5-siRNA (809) has the targeting sequence of gtagctctaacaggttgga to the 809–827 nucleotide positions. The targeting sequence for lacZ-siRNA is gtgaccagcgaatacctgt, which is directed to the 1915–1933 region of the bacterial galactosidase gene.

FG12 lentiviral vector was derived from FUGW (26). The extra nucleotides from the *Hin*dIII site downstream of the Ubiquitin-C promoter (UbiC) promoter to the *Nco*I site in front of the initiation codon of GFP were deleted by a *Hin*dIII–*Nco*I adapter ligation. Further, *Xba*I, *Eco*RI, and *Xho*I sites at the 3′ end of GFP and WRE were eliminated, followed by a polylinker oligonucleotide ligation at the *Pac*I site between the Flap element and the UbiC promoter, to generate a set of new restriction sites, *Xba*I–*Hpa*I–*Xho*I–*Bst*XI–*Pac*I, that is optimal for accommodating the siRNA expression cassette (X.-F.Q. and D.B., unpublished data). To construct the siRNA-expressing lentiviral vectors, the siRNA expression cassette was subcloned into FG12 between the *Xba*I and *Xho*I sites. The resulting plasmid was confirmed by restriction enzyme digestion and DNA sequencing.

Abbreviations: siRNA, small interfering RNA; PBLs, peripheral blood lymphocytes; FACS, fluorescence-activated cell sorter; HSA, murine heat-stable antigen; UbiC, Ubiquitin-C promoter.

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Lentiviral Vector Production. All vesicular stomatitis virus (VSV)-G pseudotyped lentiviral vector stocks were produced by calcium phosphate-mediated transient transfection of HEK-293 T cells. Briefly, HEK-293 T cells were cultured in either Iscov's modified Eagle's medium or DMEM (GIBCO Invitrogen) containing 10% FCS (HyClone), 100 units of penicillin, and 100 μg/ml streptomycin. The cells were cotransfected with appropriate amounts of vector plasmid, the HIV-1 lentiviral packaging constructs pRSVREV (27) and pMDLg/pRRE (27), and the VSV-G expression plasmid pHCMVG (28). The viruses were collected from the culture supernatants on days 2 and 3 post-transfection and concentrated 100- to 1,000-fold by ultracentrifugation (28). The concentrated virus stocks were titered on HEK-293 T cells based on GFP expression. Titers for the siRNA expression constructs were only slightly reduced compared with the parental vector.

Cell Culture and Lentiviral Vector Transduction. Magi-CCR5 cells (29) (AIDS Research and Reference Reagent Program of the National Institutes of Health) were maintained in DMEM, 10% FCS containing 200 μg of G418 (GIBCO/BRL), 100 μg of hygromycin, and 1 μg/ml puromycin (Sigma). The stable expression of human CD4 and CCR5 on the cell surface was confirmed by fluorescence-activated cell sorter (FACS) analysis. The cells were transduced with concentrated lentiviral vector stocks at a multiplicity of infection (moi) of 10–25 (note that Magi-CCR5 cells have a considerable lower transducing efficiency) in the presence of 8 μg/ml polybrene (Sigma). The transduced cells were harvested 4 days later and stained with Cy-Chrome-labeled mouse anti-human CCR5 mAb (2D7, PharMingen) or a mouse IgG2a/k isotype control (OX-35, PharMingen), according to the manufacturer's instructions. Human peripheral blood lymphocytes (PBLs) were isolated from leukopacks by Histopaque (Sigma) and cultured in RPMI medium 1640/20% FCS with 2.5 μg of phytohemagglutinin (PHA) (Murex Biotech, Dartford, U.K.)/100 units of penicillin/100 μg/ml streptomycin for 2 days. After 2 days of PHA stimulation, CD8+ cells were depleted by M450 CD8 Dynabeads (Dynal, Great Neck, NY) and the residual amount of CD8+ cells was <1%, as confirmed by FACS analysis. The CD8+-depleted PBLs were used for lentiviral vector transduction. Briefly, 4×10^5 cells were incubated with various lentiviral vectors at a moi of 5 for 2 h in the presence of 8 μg/ml polybrene. After the incubation, virus supernatants were removed and replaced with 1.5 ml of fresh RPMI medium 1640/20% FCS containing 20 units/ml IL-2 (Roche Molecular Biochemicals). GFP, CD4, CCR5, and CXCR4 expression was analyzed by FACS at multiple time points after transduction. Note that, although a moi of 5 was used for transduction with different lentivectors, actual transduction efficiency appeared to vary from sample to sample, depending on the initial titers of the virus preparations.

HIV-1 Virus Production and Infection. Stocks for the murine heat-stable antigen (HSA)-expressing HIV-1 reporter viruses, NFNSX-r-HSAS (CCR5-tropic; ref. 30) and NL-r-HSAS (CXCR4-tropic; ref. 31), were produced by calcium phosphate transfection with the infectious proviral plasmid in HEK-293 T cells. The virus supernatants were filtered with 0.22-μm filters and stored at −70°C. The p24 value of the virus stocks was 6,966 ng/ml for NFNSX-r-HSAS and 1,077 ng/ml for NL-r-HSAS. Four days after lentivector or mock transduction, the PBLs (5×10^5 cells) were infected with 100 μl of NFNSX-r-HSAS or NL-r-HSAS virus in the presence of 8 μg/ml polybrene for 2 h. After the incubation, the cells were washed and replated with 1.5 ml of RPMI medium 1640/20% FCS and 20 units/ml IL-2. The rate of infection was determined by FACS analysis for HSA expression on the cell surface at various times points as indicated. p24 levels in the culture supernatants were measured by ELISA.

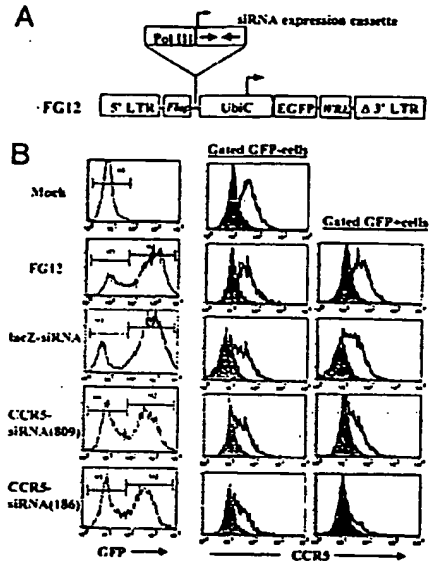

Fig. 1. Construction of a lentivirus-based vector for delivering anti-human CCR5 siRNAs. (*A*) Schematic diagram of the siRNA-expressing lentiviral vector. The short hairpin form of siRNA is expressed under the control of a human U6-RNA Pol III promoter (Pol III). The vector also contains a human UbiC promoter driving the GFP marker gene for tracking transduced cells. 5' LTR, HIV-1 5'LTR; Δ3' LTR, HIV-1 self-inactivating 3'LTR; *Flap*, HIV-1 DNA flap element; *WRE*, woodchuck hepatitis B virus RNA regulatory element. (*B*) Selection of siRNA constructs that can effectively inhibit CCR5 expression in Magi-CCR5 cells. Magi-CCR5 cells were transduced with various lentiviral vectors. The cells were harvested 4 days after virus transduction and analyzed by FACS with anti-human CCR5 or isotype control antibody staining. The productively transduced (GFP+) and nontransduced (GFP−) cells were gated based on their GFP signals. The CCR5 staining is represented by the open curve and the isotype control by the shaded curve. Note that some lower GFP-expressing cells were included in the GFP− gate and, thus, there appeared to be a slight decrease of CCR5 staining in this population of the anti-CCR5 vector-transduced samples. CCR5-siRNA(186), the vector expressing a potent anti-CCR5 siRNA; CCR5-siRNA(809), the vector expressing a weaker anti-CCR5 siRNA; lacZ-siRNA, a nonspecific siRNA vector; FG12, empty vector; Mock, mock transduction control.

FACS Analysis. Cells (5×10^5) were stained with monoclonal antibodies to human CCR5 [2D7, allophycocyanin (APC)-labeled, BD Biosciences], CXCR4 (CXCR4, APC-labeled, BD Biosciences), and CD4 [RPAT4, phycoerythrin (PE)-labeled, eBioscience], according to the manufacturer's instructions. For measuring HIV-1 reporter virus infection, a PE-labeled anti-murine HSA mAb (M1/69, PharMingen) was used. The cells were also stained with isotype controls for each of the specific antibodies. The stained cells were fixed with 2% formaldehyde and acquired on a FACScan or FACSCalibur (Becton Dickinson). The data analysis was performed with CELLQUEST (Becton Dickinson) or FLOWJO (Tree Star, San Carlos, CA) software.

Results

The backbone of the siRNA expression vector was derived from FUGW, an HIV-1-based lentivirus vector originally designed for germ-line gene transduction (26). The short hairpin form of siRNA template was transcribed from a human U6-RNA polymerase III (Pol III) promoter, and the expression cassette was inserted in the forward orientation at the junction of the HIV-1 DNA Flap element (Flap) and UbiC of the vector FG12 (Fig.

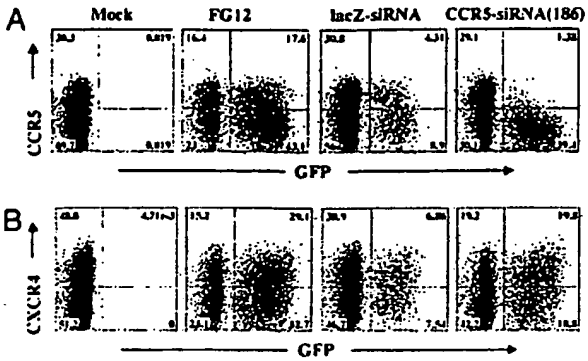

Fig. 2. Reduction of CCR5 surface expression on human PBLs transduced by anti-CCR5 lentiviral vector. PHA-stimulated and CD8+-depleted PBLs were transduced with various lentiviral vectors as described in Fig. 1. The transduced cells were further cultured in IL-2-containing medium for 8 days before FACS analysis for CCR5 or CXCR4 expression on the cell surface. A representative experiment with Donor B is shown. (*A*) Cells were stained with allophycocyanin (APC)-labeled anti-human CCR5 mAb (2D7, PharMingen). The results are exhibited as CCR5 vs. GFP dotplots with cell populations in the live lymphocyte gate (typically >75%). The quadrant lines were defined by mock-transduction and isotype-control staining, and the percentage numbers are indicated. (*B*) Cells were stained with APC-labeled anti-human CXCR4 mAb (CXCR4, PharMingen), and the results are plotted in the same manner as in *A*.

1*A*). This configuration permits high-titer virus production and the functional expression of siRNA templates in murine splenocytes, bone marrow cells, and transgenic animals (X.-F.Q., C. Lois, and D.B., unpublished data). The vector also expresses a human UbiC-driven GFP gene to provide a marker for tracking transduced cells (Fig. 1*A*). To identify an effective siRNA targeted to CCR5, we first used Magi-CCR5 cells (29) carrying a transfected human CCR5 gene as a target. Mock-transduced Magi-CCR5 cells expressed no GFP but had a high level of CCR5 expression on the cell surface (Fig. 1*B*). Cells transduced by a vector lacking the siRNA cassette (FG12) or a control siRNA cassette against lacZ (lacZ-siRNA) contained many GFP+ cells (positively transduced cells), but CCR5 expression was unaltered in these cells (Fig. 1*B*). However, with an anti-CCR5-specific siRNA-expressing vector targeting to the 809–827 region of the human CCR5 mRNA coding sequence [CCR5-siRNA (809)], the surface expression of CCR5 on the GFP+ cells was dropped to 30–40% the level of the controls, judging by the mean fluorescence intensity of CCR5 antibody staining (Fig. 1*B*). More significantly, the anti-CCR5 siRNA construct directed to the 186–204 region [CCR5-siRNA (186)] resulted in >90% reduction of CCR5 expression (Fig. 1*B*). In addition to CCR5-siRNA (809) and CCR5-siRNA (186), we also tested several other constructs targeting to different regions of CCR5 mRNA; these exhibited variable degrees of effectiveness but none >90% inhibition (not shown). A systematic approach might in the future identify even more potent siRNA templates, but the 90% inhibition provided by the CCR5-siRNA (186) vector appeared to be sufficient for our further studies described below.

To test the efficacy of the vector against HIV-1 infection in primary human $CD4^+$ T cells, PBLs depleted of CD8+ cells were isolated, stimulated with phytohemagglutinin (PHA) for 2 days, and transduced or mock transduced with various vectors. After the transduction, the cells were cultured in the presence of human IL-2. IL-2 elicits the synthesis and surface expression of CCR5 as well as the other major HIV-1 coreceptor CXCR4 on activated T cells (32, 33). We monitored CCR5 and CXCR4 expression levels at various time points by FACS. Fig. 2 shows FACS plots from a representative experiment (Donor B) at day 8 post-vector transduction. Quantitative analyses of the FACS results from two donors (A and B) are shown in Table 1, and more extensive time course studies with four different donors (A, B, C, and D) will be presented in Table 3. The mock-transduced cells in Fig. 2*A* showed that ≈30% of the cells were CCR5+. The cells transduced with the vector control (FG12) had ≈60% GFP+, and among those positively transduced cells, there were still ≈29% cells expressing CCR5 (Table 1, Donor B). lacZ-siRNA-transduced cells had a similar percentage of CCR5 positivity (Table 1), but there were fewer GFP positive cells, presumably because of a lower titer of the viral stock. Over 40% of PBLs were productively transduced by the CCR5-siRNA (186) vector, and within this GFP+ population only 3.4% of the cells scored positive for CCR5 expression, suggesting that siRNA expression caused an ≈10-fold reduction in CCR5 positivity compared with a lacZ-siRNA control (Table 1). Of the remaining CCR5-siRNA (186)-transduced cells that still expressed CCR5, the mean fluorescence intensity as a measure of relative abundance of CCR5 surface expression was substantially reduced. For the GFP− population, levels of CCR5 expression were slightly higher than in controls (Table 1), showing that down-regulation of CCR5 expression only occurred in the productively transduced cells. In contrast to CCR5, Fig. 2*B* and

Table 1. Selective down-regulation of CCR5 expression by lentiviral vector-mediated delivery of anti-CCR5 siRNA in human PBLs

		CCR5 expression			CXCR4 expression		
Donor	Treatment*	% of CCR5+ cells in the GFP− population†	% of CCR5+ cells in the GFP+ population†	Fold of reduction‡	% of CXCR4+ cells in the GFP− population†	% of CXCR4+ cells in the GFP+ population†	Fold of reduction‡
A	Mock	36.83	N/A		37.43	N/A	
	FG12	43.03	27.96		37.07	46.38	
	lacZ-siRNA	50.20	25.36		33.94	49.24	
	CCR5-siRNA	42.87	3.63	6.99	39.95	52.22	0.94
B	Mock	30.30	N/A		48.80	N/A	
	FG12	41.62	29.00		39.68	47.09	
	lacZ-siRNA	35.48	32.63		45.44	47.64	
	CCR5-siRNA	49.16	3.38	9.65	31.27	51.30	0.93

N/A, not applicable.

*Lentiviral vector transduction was performed as described in Fig. 2. Eight days after the transduction, the cells were analyzed by FACS for the surface expression of CCR5 or CXCR4 (*Materials and Methods*).

†Calculated from the quadrant percentage numbers from the CCR5 or CXCR4 vs. GFP FACS plots shown in Fig. 2.

‡Expressed as the ratio of the percentage of CCR5+ or CXCR4+ cells scored in the GFP+ population between lacZ-siRNA and CCR5-siRNA [CCR5-siRNA(186)]-transduced samples.

Table 2. Inhibition of CCR5-tropic HIV-1 infection by lentiviral vector-mediated delivery of anti-CCR5 siRNA in human PBLs

Donor	Treatment*	Response to CCR5-tropic HIV-1 challenge: % of HSA+ cells in the GFP− population†	% of HSA+ cells in the GFP+ population†	Fold of inhibition‡	Response to CXCR4-tropic HIV-1 challenge: % of HSA+ cells in the GFP− population†	% of HSA+ cells in the GFP+ population†	Fold of inhibition‡
A	Mock	4.07	N/A		4.82	N/A	
	FG12	4.26	5.64		7.26	8.70	
	lacZ-siRNA	4.16	5.39		7.94	8.33	
	CCR5-siRNA	2.02	1.48	3.64	5.90	5.76	1.45
B	Mock	5.05	N/A		6.07	N/A	
	FG12	7.16	7.95		5.15	5.78	
	lacZ-siRNA	7.98	11.08		7.76	8.15	
	CCR5-siRNA	2.55	1.73	6.41	6.91	7.41	1.10

N/A, not applicable.

*The lentivector-transduced PBLs were challenged with the CCR5- or CXCR4-tropic reporter HIV-1 viruses as described in Fig. 3. Four days after virus challenge, the cells were analyzed by FACS for HSA expression to determine the number of cells infected by the HIV-1 virus (*Materials and Methods*).

†Calculated from the quadrant percentage numbers from the HSA vs. GFP FACS plots shown in Fig. 3.

‡Expressed as the ratio of the percentage of HSA+ cells in the GFP+ population between lacZ-siRNA and CCR5-siRNA [CCR5-siRNA(186)]-transduced samples.

the data in Table 1 show that CXCR4 expression was not affected by the anti-CCR5 siRNA-expressing vector. Thus, lentivector-mediated expression of an appropriate siRNA in primary human T cells can specifically reduce the expression of CCR5.

To examine the effect of blocking CCR5 expression by the anti-CCR5 siRNA lentivector on HIV-1 infection, the lymphocyte populations were challenged with a CCR5-tropic HIV-1 reporter virus. This reporter virus is modified to express the HSA marker in place of the HIV-1 accessory gene Vpr, which allows the assay for HIV-1 infection at the single-cell level by enumerating HSA+ cells (30). Table 2 shows the quantitative results of FACS analysis with two different donor samples and Fig. 3 shows representative FACS plots from Donor B. With the empty vector (FG12) and the lacZ-siRNA vector (lacZ-siRNA), 8% and 11% of the GFP+ cells were infected by the virus, as measured by the expression of the HSA marker (see Table 2 for percentages). With the anti-CCR5 siRNA vector [CCR5-siRNA (186)] treatment, only 1.7% of the GFP+ cells were detectably positive for HIV-1 infection, Thus, for this donor, expression of the specific siRNA led to a >6-fold drop in the level of cells infected by a CCR5-tropic HIV-1 virus. A slightly lower level of protection (3.64-fold) was also obtained from another donor (Table 2, Donor A). More extensive time course experiments with additional donors (Table 3) showed that effective CCR5 down-regulation and inhibition to CCR5-tropic HIV-1 infection occurred with all of the donors over a period of 7–8 days, although the degree of effectiveness varied from donor to donor (within the range of 3- to 7-fold of protection). In addition to the GFP+-transduced cells, we found that the frequency of the HIV-1-infected HSA+ cells in the GFP− population was also decreased 2- to 3-fold in comparison with the mock, empty vector (FG12), and lacZ-siRNA controls (Fig. 3*A*; Table 2, Donors A and B). Consistent with this observation, supernatant p24 levels, a measurement of virus production, were reduced ≈3-fold (Fig. 4), despite the fact that cells without siRNA to CCR5 outnumber the siRNA-transduced population. Thus, the anti-CCR5 siRNA was not only effective at protecting the transduced cells in a mixed population, but also resulted in an overall reduction of virus load and decreased infection of

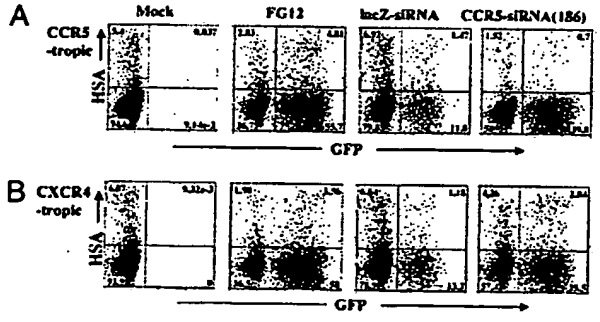

Fig. 3. Inhibition of CCR5-tropic HIV-1 infection in human PBLs transduced by lentiviral vector expressing anti-CCR5 siRNA. PBLs were transduced by various lentiviral vectors as described in Fig. 2. The transduced PBLs were cultured in IL-2-containing medium for 4 days before being challenged with either the CCR5-tropic reporter virus (*A*) or the CXCR4-tropic reporter virus (*B*). The cells were harvested 4 days after the virus challenge, and the virus-infected cells were quantitated by FACS analysis for the expression of the HSA marker. The FACS results are presented as HSA vs. GFP dotplots with cell populations in the live lymphocyte gate. The quadrant lines were defined by mock-infection and isotype-control staining, and the percentage numbers are indicated. One representative experiment with Donor B is shown.

Table 3. Time course study on the reduction of CCR5 expression and inhibition to CCR5-tropic HIV-1 infection with the CCR5-siRNA lentivector-transduced PBLs from different donors

Donor	Days after HIV-1 challenge	Reduction of CCR5	HIV-1 inhibition
A	4	6.99	3.64
	7	6.61	2.64
B	4	9.65	6.41
	6	10.54	7.04
	8	4.88	5.81
C	4	10.09	1.25
	6	8.7	3.48
	8	9.56	3.73
D	4	13.26	2.59
	6	13.75	3.68
	8	5.68	4.09

CD8+-depleted PBLs from four different donors were transduced with various lentiviral vectors, as described in Fig. 2. Four days after the transduction, the cells were challenged with the CCR5-tropic HIV-1 reporter virus (as in Fig. 3). The virus-infected cells were analyzed by FACS for CCR5 and HSA expression at the different time points indicated. The reduction of CCR5 expression and inhibition of CCR5-tropic HIV-1 infection were determined in the same manner as in Tables 1 and 2. Equivalent degrees of CCR5 down-regulation were obtained with the similarly transduced cells but not being challenged with the HIV-1 virus.

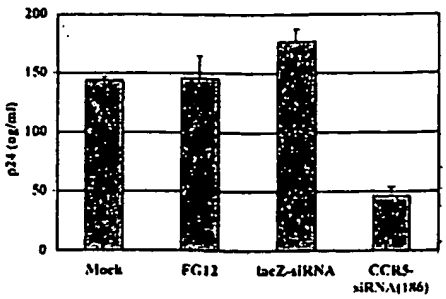

Fig. 4. Decrease of p24 production in HIV-1-infected cultures of anti-CCR5 lentiviral vector-transduced human PBLs. Culture supernatants were collected 6 days after the HIV-1 virus challenge, as described in Fig. 3, and p24 levels were measured by ELISA in triplicates. Again, the representative result from Donor B is shown.

nontransduced cells over the course of HIV-1 infection. As a control, the lymphocyte populations were also infected with a CXCR4-tropic HIV-1 reporter virus similarly modified to express HSA (31). Infection of the CXCR4-tropic virus was not significantly inhibited by the transduction of an anti-CCR5 siRNA vector (Fig. 3*B*, Table 2), confirming the specificity of the siRNA-mediated inhibition. Taken together, we conclude that a lentivirus vector expressing a potent siRNA against CCR5 can substantially reduce CCR5 expression on the cell surface in a specific manner and render the transduced cells relatively resistant to CCR5-tropic HIV-1 infection.

Discussion

These results demonstrate that an siRNA directed to a cellular gene that is required for HIV-1 infection, namely CCR5, can inhibit HIV-1 replication. With HIV-1 replication as a model system, our studies also demonstrate the power and general utility of using lentiviral vectors for the stable expression of siRNAs in primary human cells to inhibit the expression of cellular genes.

The human chemokine receptor gene, CCR5, was chosen as a target for these studies for several reasons. First, CCR5 is a necessary coreceptor for infection by most strains of HIV-1 (34, 35). After binding of the HIV-1 envelope protein gp120 to CD4, interaction with gp120 and CCR5 induces a conformational change that then leads to HIV-1 envelope gp41 fusion with the cell membrane (36). This is an obligatory step for the infection process of CCR5-tropic strains of HIV-1; the absence of CCR5 prevents HIV-1 infection to cells. Not all strains of HIV-1 require CCR5. CXCR4, for example, is another major coreceptor (37). However the majority of naturally occurring strains of HIV-1 use CCR5 as a coreceptor for primary infection (38–41). Second, CCR5 is apparently dispensable for normal human growth, differentiation, and immune functions (42). Among the white population, the occurrence of a CCR5-null allele, known as CCR5Δ32, is ≈1% (21–24). The CCR5Δ32 allele is a deletion resulting in a frameshift truncation of CCR5 that prevents the mutant protein from appearing on the cell surface (21, 33). Individuals homozygous for the CCR5Δ32 do not show any apparent adverse phenotypic effects, although a recent report linked CCR5Δ32 homozygosity with higher viral loads in hepatitis C infection (43). In heterozygous CCR5Δ32 individuals, cell surface CCR5 is reduced to 20–30% of wild-type levels (33, 44, 45). Again, no adverse effects are observed in these individuals (42). It will be important to confirm that acquired loss of CCR5 expression through siRNA inhibition also has no adverse phenotypic effects.

Cohort studies indicate that individuals that are homozygous for the CCR5 mutation are almost completely resistant to infection by CCR5-tropic strains of HIV-1 (38–41, 46, 47). To date, there have been only eight reports of HIV-1 infection in CCR5Δ32 homozygous individuals (48–51). These individuals appeared to have been infected with a CXCR4-tropic strain of HIV-1 and did develop AIDS. Individuals that are heterozygous for CCR5Δ32 are infected at rates similar to CCR5+/CCR5+ individuals. However, their disease course is prolonged, presumably because of reduced spread of virus within the individuals over time (23). Thus, the 3- to 7-fold inhibition produced by our anti-CCR5 siRNA vector could already have a marked clinical effect and, with an optimized system, a greater inhibition might even be obtained. However, it is possible that in a patient producing HIV-1, anti-CCR5 siRNA could select for viral variants that use CXCR4 and then cause progression to AIDS. Therefore, a CCR5 inhibitory therapy would be best used in combination with other antiviral approaches.

Because of the importance of CCR5 to HIV-1 infection and disease development, a number of approaches have been investigated to inhibit the utilization of CCR5 as a coreceptor by HIV-1. The chemokines, RANTES, MIP-1α, and MIP1-β, are physiological ligands for CCR5 and have been shown to inhibit HIV-1 infection both *in vitro* and *in vivo* (52). However, these chemokines and their pharmacological derivatives have not been used clinically because they have a short half-life, are not orally bioavailable, and may have other effects (53, 54). Other investigators have inhibited CCR5 *in vitro* by using "intrakines," fusions of RANTES and MIP1-α to endoplasmic reticulum retention signals (55, 56), and single-chain antibodies directed to CCR5 (30).

We have shown here that it is possible to obtain at least partial inhibition of a viral infection by lentiviral vector-mediated expression of an siRNA targeting a cellular gene. Within the transduced population of T cells (GFP+ fraction), we observed an up to 10-fold inhibition of CCR5 expression. As a consequence, HIV-1 infection within the transduced population was also substantially reduced, relative to mock-transduced cells, cells transduced with an irrelevant siRNA (lacZ-siRNA), or nontransduced cells in the same culture (GFP− fraction). However, there remained transduced cells that were infected by HIV-1 (HSA+). Residual infection of transduced cells may occur because of incomplete inhibition, variability of inhibition over time, or infection through a CCR5-independent mechanism (57). It is important to remember that in this culture system, the cells are bathed continuously in virus produced by the unprotected cells.

Although a 3-fold reduction of the total virus load in our *in vitro* model is rather modest, an effective clinical application of this technology would be through a hematopoietic stem cell transplant. In this manner, therapeutic siRNA-expressing stem cells would give rise to mature progeny T cells, macrophages, and/or dendritic cells that would be relatively protected from infection by HIV-1. Furthermore, the rapid demise of HIV-1-infected T cells (58) should lead to rapid selection for the CCR5-negative cells that are relatively protected from the effects of HIV-1. We did not observe selection in cell culture, possibly because the HIV-1 viruses that we used are lacking the Vpr gene as a result of substitution by the HSA reporter gene. Vpr induces cell-cycle arrest, followed by apoptosis, which would lead to rapid selection against HIV-1-infected cells (59). Selection of transduced cells expressing a therapeutic transgene has played an important role in the successful human clinical trials of gene therapy, the treatment of X-linked SCID-X1 and ADA-SCID genetic disorders (60, 61).

In summary, our studies provide proof of principle for the idea that siRNA directed to a gene essential for viral replication can serve as a potential therapeutic agent for human infectious disease. The utilization of siRNA as a therapeutic reagent in the clinical setting is advantageous to previously reported ap-

proaches in that siRNA is a small nucleic acid reagent as opposed to relatively large chimeric or antibody proteins. A small nucleic acid reagent should be less likely to elicit an immune response. Furthermore, the relative simplicity of the siRNA approach lends itself to easy combination with other siRNAs directed to different regions of CCR5 and/or other cellular or HIV-1-specific genes, which can result in additive or synergistic effects, and will help to prevent the escape of mutant variants. It seems possible that further modifications in vector design and choice of siRNAs simultaneously targeting multiple essential components for viral replication could provide an effective treatment against HIV-1.

We thank Carlos Lois, Yi-ming Xie, Si Hua Mao, Vaheideh Gudeman, and Kathie Grovit-Ferbas for reagents and helpful discussions. This work was supported by grants from the National Institutes of Health to D.B. (AI42549-04) and I.S.Y.C. (AI39975-05 and AI28697). X.-F.Q. was supported by Damon Runyon–Walter Winchell Fellowship DRG-1568.

1. Baltimore, D. (1988) *Nature* 335, 395–396.
2. Sharp, P. A. (2001) *Genes Dev.* 15, 485–490.
3. Tuschl, T. (2002) *Nat. Biotechnol.* 20, 446–448.
4. Zamore, P. D. (2002) *Science* 296, 1265–1269.
5. Hannon, G. J. (2002) *Nature* 418, 244–251.
6. Brummelkamp, T. R., Bernards, R. & Agami, R. (2002) *Science* 296, 550–553.
7. Paddison, P. J., Caudy, A. A., Bernstein, E., Hannon, G. J. & Conklin, D. S. (2002) *Genes Dev.* 16, 948–958.
8. Yu, J. Y., DeRuiter, S. L. & Turner, D. L. (2002) *Proc. Natl. Acad. Sci. USA* 99, 6047–6052.
9. Sui, G., Soohoo, C., Affar el, B., Gay, F., Shi, Y., Forrester, W. C. & Shi, Y. (2002) *Proc. Natl. Acad. Sci. USA* 99, 5515–5520.
10. Miyagishi, M. & Taira, K. (2002) *Nat. Biotechnol.* 20, 497–500.
11. Paul, C. P., Good, P. D., Winer, I. & Engelke, D. R. (2002) *Nat. Biotechnol.* 20, 505–508.
12. Naldini, L. & Verma, I. M. (2000) *Adv. Virus Res.* 55, 599–609.
13. Kay, M. A., Glorioso, J. C. & Naldini, L. (2001) *Nat. Med.* 7, 33–40.
14. An, D. S., Wersto, R. P., Agricola, B., Metzger, M., Lu, S., Amado, R. G., Chen, I. S. Y. & Donahue, R. E. (2000) *J. Virol.* 74, 1286–1295.
15. An, D. S., Kung, S. K., Bonifacino, A., Wersto, R. P., Metzger, M. E., Agricola, B. A., Mao, S. H., Chen, I. S. & Donahue, R. E. (2001) *J. Virol.* 75, 3547–3555.
16. Lee, N. S., Dohjima, T., Bauer, G., Li, H., Li, M. J., Ehsani, A., Salvaterra, P. & Rossi, J. (2002) *Nat. Biotechnol.* 20, 500–505.
17. Novina, C. D., Murray, M. F., Dykxhoorn, D. M., Beresford, P. J., Riess, J., Lee, S. K., Collman, R. G., Lieberman, J., Shankar, P. & Sharp, P. A. (2002) *Nat. Med.* 8, 681–686.
18. Jacque, J. M., Triques, K. & Stevenson, M. (2002) *Nature* 418, 435–438.
19. Coburn, G. A. & Cullen, B. R. (2002) *J. Virol.* 76, 9225–9231.
20. Hu, W., Myers, C., Kilzer, J., Pfaff, S. & Bushman, F. (2002) *Curr. Biol.* 12, 1301–1311.
21. Liu, R., Paxton, W. A., Choe, S., Ceradini, D., Martin, S. R., Horuk, R., MacDonald, M. E., Stuhlmann, H., Koup, R. A. & Landau, N. R. (1996) *Cell* 86, 367–377.
22. Samson, M., Libert, F., Doranz, B. J., Rucker, J., Liesnard, C., Farber, C. M., Saragosti, S., Lapoumeroulie, C., Cognaux, J., Forceille, C., *et al.* (1996) *Nature* 382, 722–725.
23. Dean, M., Carrington, M., Winkler, C., Huttley, G. A., Smith, M. W., Allikmets, R., Goedert, J. J., Buchbinder, S. P., Vittinghoff, E., Gomperts, E., *et al.* (1996) *Science* 273, 1856–1862.
24. Huang, Y., Paxton, W. A., Wolinsky, S. M., Neumann, A. U., Zhang, L., He, T., Kang, S., Ceradini, D., Jin, Z., Yazdanbakhsh, K., *et al.* (1996) *Nat. Med.* 2, 1240–1243.
25. Paule, M. R. & White, R. J. (2000) *Nucleic Acids Res.* 28, 1283–1298.
26. Lois, C., Hong, E. J., Pease, S., Brown, E. J. & Baltimore, D. (2002) *Science* 295, 868–872.
27. Dull, T., Zufferey, R., Kelly, M., Mandel, R. J., Nguyen, M., Trono, D. & Naldini, L. (1998) *J. Virol.* 72, 8463–8471.
28. Yee, J. K., Friedmann, T. & Burns, J. C. (1994) *Methods Cell Biol.* 43, 99–112.
29. Chackerian, B., Long, E. M., Luciw, P. A. & Overbaugh, J. (1997) *J. Virol.* 71, 3932–3939.
30. Steinberger, P., Andris-Widhopf, J., Buhler, B., Torbett, B. E. & Barbas, C. F., III (2000) *Proc. Natl. Acad. Sci. USA* 97, 805–810.
31. Jamieson, B. D. & Zack, J. A. (1998) *J. Virol.* 72, 6520–6526.
32. Bleul, C. C., Wu, L., Hoxie, J. A., Springer, T. A. & Mackay, C. R. (1997) *Proc. Natl. Acad. Sci. USA* 94, 1925–1930.
33. Wu, L., Paxton, W. A., Kassam, N., Ruffing, N., Rottman, J. B., Sullivan, N., Choe, H., Sodroski, J., Newman, W., Koup, R. A. & Mackay, C. R. (1997) *J. Exp. Med.* 185, 1681–1691.
34. Alkhatib, G., Combadiere, C., Broder, C. C., Feng, Y., Kennedy, P. E., Murphy, P. M. & Berger, E. A. (1996) *Science* 272, 1955–1958.
35. Dragic, T., Litwin, V., Allaway, G. P., Martin, S. R., Huang, Y., Nagashima, K. A., Cayanan, C., Maddon, P. J., Koup, R. A., Moore, J. P. & Paxton, W. A. (1996) *Nature* 381, 667–673.
36. Berger, E. A. (1997) *AIDS* 11, S3–S16.
37. Feng, Y., Broder, C. C., Kennedy, P. E. & Berger, E. A. (1996) *Science* 272, 872–877.
38. Zhu, T., Mo, H., Wang, N., Nam, D. S., Cao, Y., Koup, R. A. & Ho, D. D. (1993) *Science* 261, 1179–1181.
39. Schuitemaker, H., Koot, M., Kootstra, N. A., Dercksen, M. W., Degoede, R. E. Y., Vansteenwijk, R. P., Lange, J. M. A., Schattenkerk, J. K. M. E., Miedema, F. & Tersmette, M. (1992) *J. Virol.* 66, 1354–1360.
40. Roos, M. T. L., Lange, J. M. A., Degoede, R. E. Y., Coutinho, R. A., Schellekens, P. T. A., Miedema, F. & Tersmette, M. (1992) *J. Infect. Dis.* 165, 427–432.
41. Richman, D. D. & Bozzette, S. A. (1994) *J. Infect. Dis.* 169, 968–974.
42. O'Brien, S. J. & Moore, J. P. (2000) *Immunol. Rev.* 177, 99–111.
43. Woitas, R. P., Ahlenstiel, G., Iwan, A., Rockstroh, J. K., Brackmann, H. H., Kupfer, B., Matz, B., Offergeld, R., Sauerbruch, T. & Spengler, U. (2002) *Gastroenterology* 122, 1721–1728.
44. Moore, J. P. (1997) *Science* 276, 51–52.
45. Benkirane, M., Jin, D. Y., Chun, R. F., Koup, R. A. & Jeang, K. T. (1997) *J. Biol. Chem.* 272, 30603–30606.
46. Zimmerman, P. A., Buckler-White, A., Alkhatib, G., Spalding, T., Kubofcik, J., Combadiere, C., Weissman, D., Cohen, O., Rubbert, A., Lam, G., *et al.* (1997) *Mol. Med.* 3, 23–36.
47. Michael, N. L., Chang, G., Louie, L. G., Mascola, J. R., Dondero, D., Birx, D. L. & Sheppard, H. W. (1997) *Nat. Med.* 3, 338–340.
48. Theodorou, I., Meyer, L., Magierowska, M., Katlama, C. & Rouzioux, C. (1997) *Lancet* 349, 1219–1220.
49. Biti, R., Ffrench, R., Young, J., Bennetts, B., Stewart, G. & Liang, T. (1997) *Nat. Med.* 3, 252–253.
50. Balotta, C., Bagnarelli, P., Violin, M., Ridolfo, A. L., Zhou, D., Berlusconi, A., Corvasce, S., Corbellino, M., Clementi, M., Clerici, M., *et al.* (1997) *AIDS* 11, F67–F71.
51. Michael, N. L., Nelson, J. A., KewalRamani, V. N., Chang, G., O'Brien, S. J., Mascola, J. R., Volsky, B., Louder, M., White, G. N., Littman, D. R., *et al.* (1998) *J. Virol.* 72, 6040–6047.
52. Cocchi, F., DeVico, A. L., Garzino-Demo, A., Arya, S. K., Gallo, R. C. & Lusso, P. (1995) *Science* 270, 1811–1815.
53. Marozsan, A. J., Torre, V. S., Johnson, M., Ball, S. C., Cross, J. V., Templeton, D. J., Quinones-Mateu, M. E., Offord, R. E. & Arts, E. J. (2001) *J. Virol.* 75, 8624–8638.
54. Simmons, G., Reeves, J. D., Hibbitts, S., Stine, J. T., Gray, P. W., Proudfoot, A. E. & Clapham, P. R. (2000) *Immunol. Rev.* 177, 112–126.
55. Yang, A. G., Bai, X., Huang, X. F., Yao, C. & Chen, S. (1997) *Proc. Natl. Acad. Sci. USA* 94, 11567–11572.
56. Schroers, R., Davis, C. M., Wagner, H. J. & Chen, S. Y. (2002) *Gene Ther.* 9, 889–897.
57. Pang, S., Yu, D., An, D. S., Baldwin, G. C., Xie, Y., Poon, B., Chow, Y. H., Park, N. H. & Chen, I. S. (2000) *J. Virol.* 74, 10994–11000.
58. Perelson, A. S., Neumann, A. U., Markowitz, M., Leonard, J. M. & Ho, D. D. (1996) *Science* 271, 1582–1586.
59. Stewart, S. A., Poon, B., Jowett, J. B. & Chen, I. S. (1997) *J. Virol.* 71, 5579–5592.
60. Cavazzana-Calvo, M., Hacein-Bey, S., de Saint Basile, G., Gross, F., Yvon, E., Nusbaum, P., Selz, F., Hue, C., Certain, S., Casanova, J. L., *et al.* (2000) *Science* 288, 669–672.
61. Aiuti, A., Slavin, S., Aker, M., Ficara, F., Deola, S., Mortellaro, A., Morecki, S., Andolfi, G., Tabucchi, A., Carlucci, F., *et al.* (2002) *Science* 296, 2410–2413.